The Journal of Biochemistry, published monthly by
the Japanese Biochemical Society, Tokyo, Japan.
Vol. 45, No. 9, September 25, 1958

Vol. 45, No. 9 **September, 1958**

THE JOURNAL

OF

BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

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THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published monthly, one volume per year.

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AEROBIC METABOLISM OF ACETIC ACID IN RHODOPSEUDOMONAS PALUSTRIS

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(Received for publication, April 28, 1958)

Acetic acid is among the most readily utilized substrates of respiration and photic assimilation of Athiorhodaceae bacteria. Its metabolism has been the subject of studies by a number of investigators since the work of van Niel (1941) (*1*), who first discovered that acetic acids was assimilated by *Rhodospirillum rubrum* either photochemically in the light or aerobically in the dark (*2*, *3*, *4*). He also found that *Rhodospirillum* oxidized acetic acid (both in the dark and in the light), only when carbon dioxide was present in the medium. He interpreted this phenomenon as being due to the "sparking" effect of C_4-dicarboxylic acids supposedly synthesized from the added carbon dioxide. This interpretation seemed to be borne out by the experiments made later by van Niel (1949) (*5*), as well as by Elsden *et al.* (1956) (*6*), who, also using *Rhodospirillum rubrum*, observed that the addition of dicarboxylic acids of the Krebs cycle was effective in producing the same effect. Using methyl- and carboxyl-labeled acetate as the substrate, Glover *et al.* (1953) (*7*) investigated the process of assimilation of acetic acid, and found that, in the light, the radiocarbon was most rapidly incorporated in the lipid fraction of cell material.

The work reported below was carried out in order to investigate, with these findings made by earlier workers in mind, the mechanism of acetate metabolism in another species of Athiorhodaceae, *Rhodopseudomonas palustris*, which has long been the object of physiological and biochemical studies in our laboratory.

MATERIALS AND METHODS

Organism—Rhodopseudomonas palustris (Molisch) Kluyver *et* van Niel, was grown under continuous illumination in a medium containing 0.5 per cent "polypeptone" and 0.5 per cent sodium lactate (pH 7.0). After 48 hours of culture at 25–27°, the cells were harvested by centrifugation, washed several times with water, and used for the experiments.

Determination of Metabolic Activities—As the substrate, carboxyl- and methyl-labeled sodium acetate (Daiichi Kagaku Co., Ltd.) was used. Most of the experiments were carried out in a Warburg vessel fitted with a stopcock at the top and with a rubber stopper at the side orifice (see the previous paper (*8*)). The center well of the vessel was filled with KOH solution for the absorption of carbon dioxide. The gas atmosphere of the vessel was always air. The reaction was started by injecting, with an injection syringe, the substrate solution through the rubber stopper into the reaction mixture. A bank of incandescent lamps (about 4,000 lux) was used as the light source. The reaction was stopped by addition of trichloracetic acid to the reaction mixture. A series of reaction vessels was used to follow the time course of reaction.

Some experiments were performed using a vessel made of Lucite, which is illustrate in Fig. 1. The reaction was started by injecting the substrate through a rubber stopper as illustrated in the figure. The reaction mixture was continuously aerated with a stream of carbon dioxide-free air; the air which passed through the reaction mixture was bubbled through a solution of KOH in a gas washing bottle to fix the carbon dioxide produced in the reaction. At appropriate intervals aliquots of reaction mixture were taken out through the rubber stopper using an injection syringe, and poured into a test tube containing glacial acetic acid to stop the reaction. In the fractionation experiments the reaction was stopped by pouring the reaction mixture into boiling methanol. All experiments were performed at 27°.

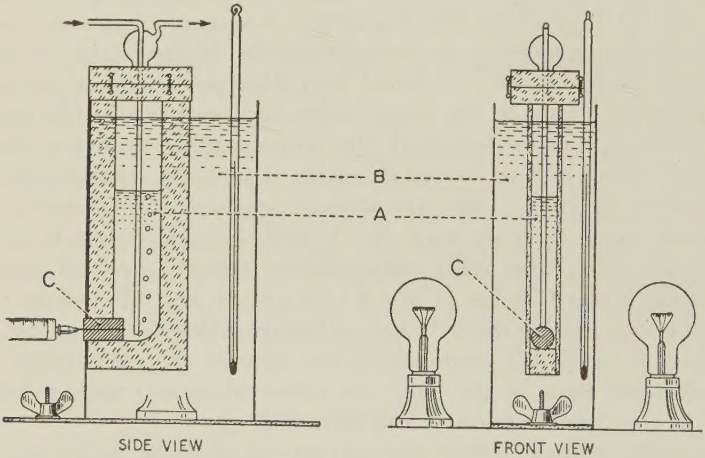

Fig. 1. Reaction vessel. A: reaction mixture; B: thermostat; C: rubber stopper. Further explanation in text.

The $C^{14}O_2$ produced during the reaction and fixed in the KOH solution was precipitated as $BaC^{14}O_3$ and its quantity was determined by measuring the radioactivity. The radioactivity of C^{14} which was incorporated in cell materials was measured with the samples which were obtained by drying aliquots of reaction mixture on a stainless steel dish with the addition of non-labeled glacial acetic acid as the carrier. For measuring radioactivity a mica-end-windowed GM-tube was used. Corrections for the salf absorption were made based on the standard curves obtained in preliminary tests. The respiratory oxygen uptake was measured with a Warburg manometer; corrections were made for the endogenous uptake of oxygen. The amounts of acetic acid in the reaction mixture were determined by the microdiffusion method after Conway.

RESULTS

Consumption and Incorporation of Acetic Acid and Gas-exchanges Involved—Carboxyl-labeled acetic acid, added to the reaction medium, was consumed without appreciable lag, both in the light and in the dark (Fig. 2). The radiocarbon metabolized was partially recovered in the cell material and partially in the carbon dioxide evolved. The time course of $C^{14}O_2$-evolution is shown in Fig. 2. As shown in the figure, the evolution of $C^{14}O_2$ always

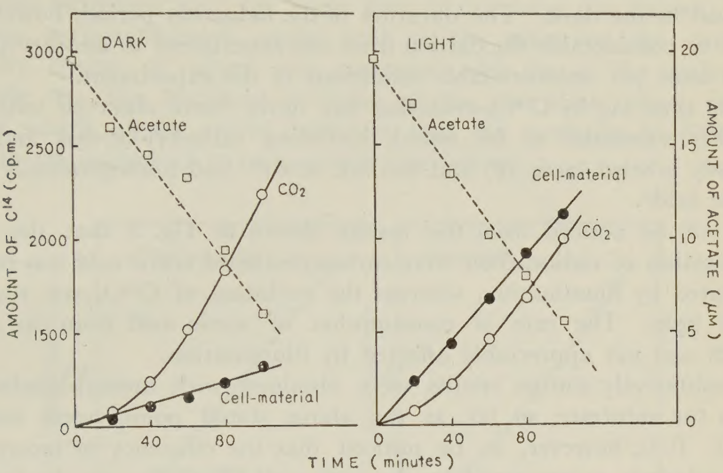

FIG. 2. Evolution of $C^{14}O_2$ and incorporation of C^{14} into cell material from carboxyl-labeled acetic acid.

Reaction vessel: Warburg's manometer vessel; bacterial cells: 44.2 mg. dry weight per vessel; substrate: carboxyl-labeled acetate, 20 μM per vessel (110 c.p.m./μM); phosphate buffer: pH 7.0, final concentration $M/50$; total volume of reaction mixture: 5 ml.

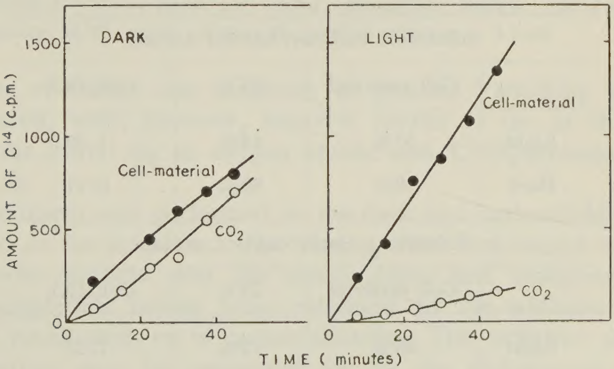

FIG. 3. Evolution of $C^{14}O_2$ and incorporation of C^{14} into cell material from methyl-labeled acetate.

Reaction vessel: Warburg's manometer vessel; bacterial cells: 12.2 mg. dry weight per vessel; substrate: methyl-labeled acetate (5 μc/mM), 10 μM per vessel; phosphate buffer: pH 7.0, final concentration $M/40$; total volume of reaction mixture: 5 ml.

occurred with a certain period of induction, a phenomenon which was in marked contrast to the fact that both the incorporation and the consumption of acetic acid occurred without any sign of an induction period. The same was also true when methyl-labeled acetic acid was used as the substrate (Fig. 3). The time lag in the evolution of $C^{14}O_2$ was observed both in the light and in the dark. The duration of the induction period, however, was subject to considerable fluctuation from one experiment to another probably due to some yet uncontrollable conditions of the experiment.

The time lag in $C^{14}O_2$-evolution has never been observed with any of the other substrates so far tested, including carboxyl-labeled and homogeneously labeled lactic (*8*) and succinic acids*, and homogeneously labeled fumaric acid*.

It will be noticed from the results shown in Fig. 2 that the rate of incorporation of radiocarbon from carboxyl-labeled acetic acid was markedly accelerated by illumination, whereas the evolution of $C^{14}O_2$ was suppressed by the light. The rate of consumption of acetic acid from the reaction medium was not appreciably affected by illumination.

Qualitatively similar results were obtained with methyl-labeled acetic acid as the substrate, so far as the above stated points were concerned (Fig. 3). It is, however, to be noticed that the efficiency of incorporation of the substrate as measured by the ratio, cell-C^{14}/$C^{14}O_2$ was by far higher with methyl-labeled acetic acid than with carboxyl-labeled acetic acid. This was found to be true irrespective of whether the experiments were performed in the light or in the dark (*cf.* Figs. 2 and 3). The ameliorative effect of light on the efficiency of C^{14}-incorporation from the added substrate has also been observed with the other substrates thus far studied (*i.e.*, lactic,

TABLE I

Evolution of $C^{14}O_2$ and Incorporation of C^{14} into Cell Material from Labeled Acetic Acid in the Dark and in the Light

Substrate : carboxyl-labeled acetate			
	Cell material	CO_2	Cell/CO_2
Light	57%	43%	1.32
Dark	18%	82%	0.21
Substrate : methyl-labeled acetate			
	Cell material	CO_2	Cell/CO_2
Light	88%	12%	7.33
Dark	53%	47%	1.33

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* Morita, S., unpublished.

succinic and fumaric acids; see above), the acceleration of incorporation and the suppression of $C^{14}O_2$-evolution always being found on illumination of the cell suspension. The relevent results are summarized in Table I. In the calculations made in these data, the values for the rate of $C^{14}O_2$-evolution were estimated from the linear part of the curve (see below).

Using carboxyl-labeled acetic acid as the substrate, the course of respiratory uptake of oxygen was compared with that of the evolution of $C^{14}O_2$ (Fig. 4). The similarity in form of the two time curves with an identical length of lag period indicates that the evolution of $C^{14}O_2$ was related to the process of oxygen uptake (see below).

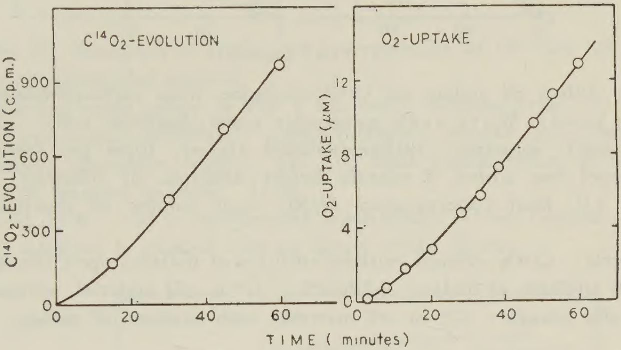


FIG. 4. $C^{14}O_2$-evolution and O_2-absorption (in the dark) as measured simultaneously with identical material.

Reaction vessel: Warburg's manometer vessel; bacterial cells: 30.6 mg. dry weight per vessel; substrate: carboxyl-labeled acetate (0.6 μc/mM) or non-labeled acetate, 50 μM per vessel; phosphate buffer: pH 7.0, final concentration *M*/35; total volume of reaction mixture: 3.5 ml.

The effect of malate and fumarate as possible "sparking agent" was also investigated, with, however, negative results so far as the intended abolition of the initial lag in oxygen uptake and $C^{14}O_2$-evolution was concerned (Fig. 5).

The experiment was performed in the dark and carboxyl-labeled acetic acid was used as the substrate. The finding does not accord with that of van Niel, who reported that the consumption and oxidation of acetic acid by *Rhodospirillum rubrum* were enhanced by the addition of succinic, fumaric, and malic acids or of carbon dioxide. The negative results with our test organism may be accounted for by the endogenous content of sufficient amounts of sparker substances.

Transitional Change in the Level of Cell-C^{14} after Exhaustion of Substrate—Using carboxyl-labeled acetate, the changes in the level of C^{14} in both the cell material and in the evolved CO_2 were followed. As shown in Figs. 6 and 7, the level of cell-C^{14} increased steadily until the bulk of the added substrate was consumed. The level then showed a distinct decrease and attained, in

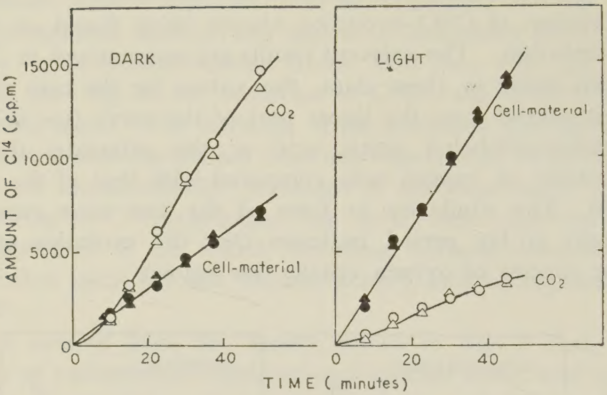


FIG. 5A. Effect of malate on $C^{14}O_2$-evolution from carboxyl-labeled acetate.

Reaction vessel: Warburg's manometer vessel; bacterial cells: 15.6 mg. dry weight per vessel; substrate: carboxyl-labeled acetate, 10 μM per vessel; malate: 0.6 μM per vessel was added 2 minutes before addition of substrate; phosphate buffer: pH 7.0, final concentration *M*/50; total volume of reaction mixture: 2.5 ml.

Open circle: $C^{14}O_2$ evolved, without addition of malate; open triangle: $C^{14}O_2$ evolved, with addition of malate; solidcircle: C^{14} in cell material, without addition of malate; solid triangle: C^{14} in cell material, with addition of malate.

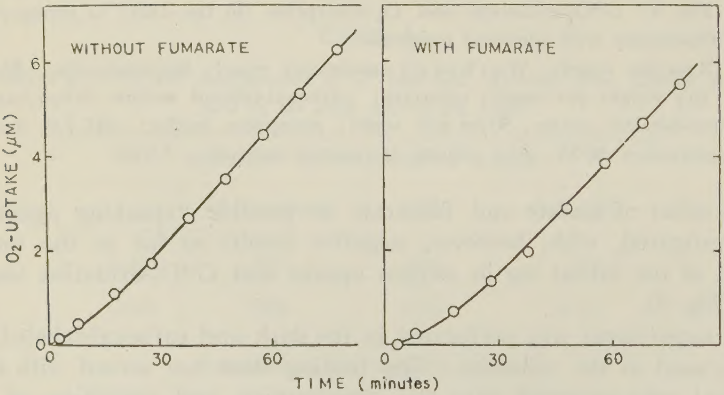


FIG. 5B. Effect of fumarate on oxygen uptake with acetate as substrate.

Reaction was measured by Warburg's manometer; bacterial cells: 12.2 mg. dry weight per vessel; substrate: non-labeled acetate, 25 μM per vessel; fumarate: 0.1 μM per vessel was added simultaneouly with substrate from the side arm of reaction vessel; phosphate buffer: pH 7.0, final concentration *M*/50; total volume of reaction mixture: 2.5 ml. Correction were made for the endogenous respiration.

a period of around half an hour, a steady value which lasted unaltered for further period of incubation. The surplus amount of incorporated radioactivity beyond the final steady level was found to be independent of the

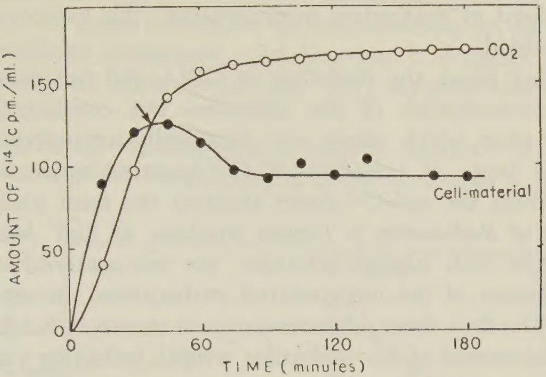


FIG. 6. Evolution of $C^{14}O_2$ and incorporation of C^{14} into cell material from carboxyl-labeled acetate.

Reaction vessel shown in Fig. 1 was used; bacterial cells: 151 mg. dry weight per vessel; substrate: carboxyl-labeled acetate (2.1 μc/mM), 50 μM per vessel; phosphate buffer: pH 7.0, final concentration $M/60$; initial volume of reaction mixture: 15 ml.

Solid circle: C^{14} in cell material; open circle: $C^{14}O_2$ evolved. Exhaustion of substrate is marked with an arrow in the figure.

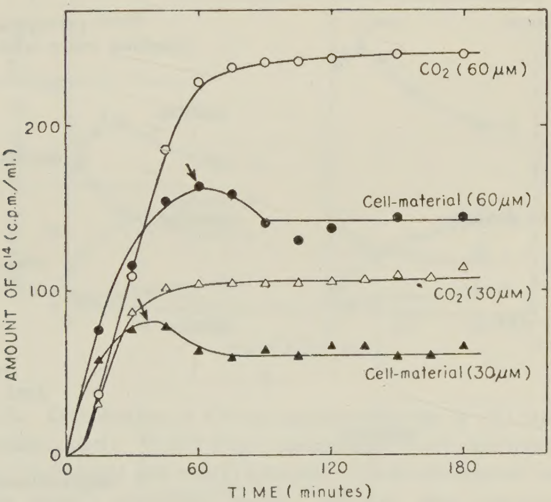


FIG. 7. C^{14}-incorporation and $C^{14}O_2$-evolution in the dark, with varied initial concentrations of substrate (carboxyl-labeled acetate).

Reaction vessel shown in Fig. 1 was used; bacterial cells: 151 mg. dry weight per vessel; substrate: carboxyl-labeled acetate (2.1 μc/mM), 30 μM and 60 μM per vessel; phosphate buffer: pH 7.0, final concentration $M/60$; initial volume of reaction mixture: 15 ml.

Open circle and open triangle: $C^{14}O_2$ evolved from 60 μM and 30 μM, respectively, of substrate; solid circle and solid triangle: C^{14} incorporated in cell material 60 μM and 30 μM, respectively, of substrate.

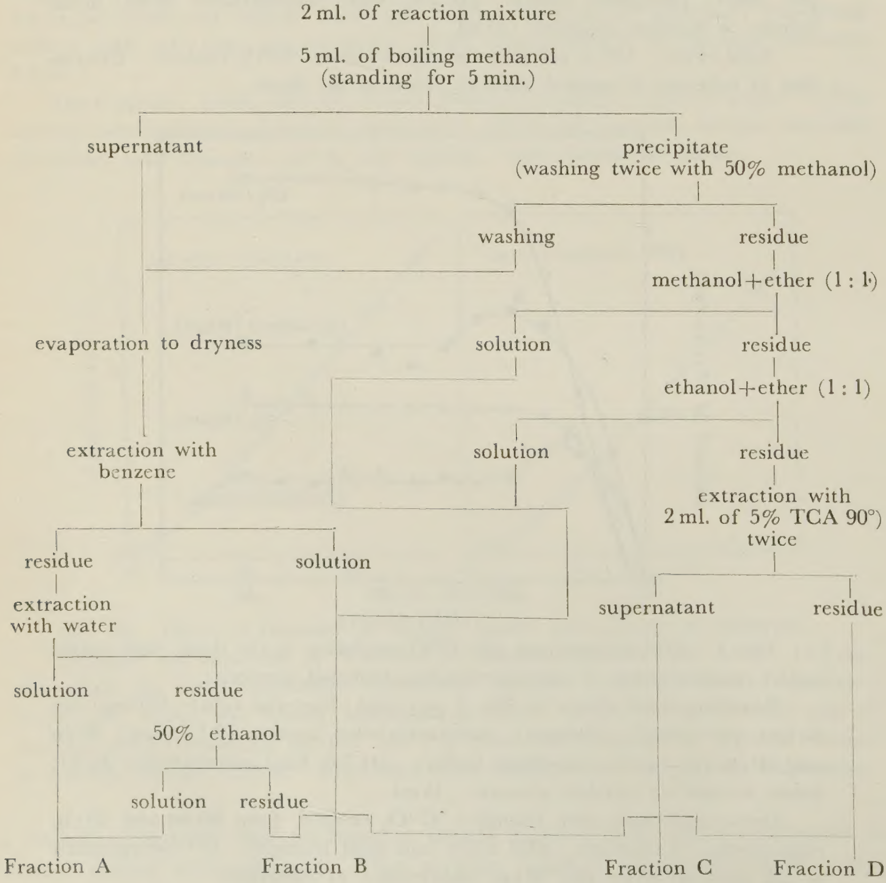
initial amonut of the substrate added, although the longer time was required for the attainment of maximum incorporation, the greater the amount of the substrate added.

On the other hand, the evolution of $C^{14}O_2$ did not come to an abrupt stop with the consumption of the substrate, but continued for a certain period of time after which there was practically no perceptible production of $C^{14}O_2$. This stage of cessation of $C^{14}O_2$-output seems to correspond to the stage, at which the cell-C^{14} curve attained the final steady level.

Distribution of Radiocarbon in Various Fractions of Cell Material—After incubating the cells with labeled substrate, the cell material was fractionated and the distribution of the incorporated radiocarbon in each fraction was investigated. The flow sheet of fractionation is shown in Scheme 1. Fraction A consists of substances of low molecular weight, including various carboxylic

SCHEME 1

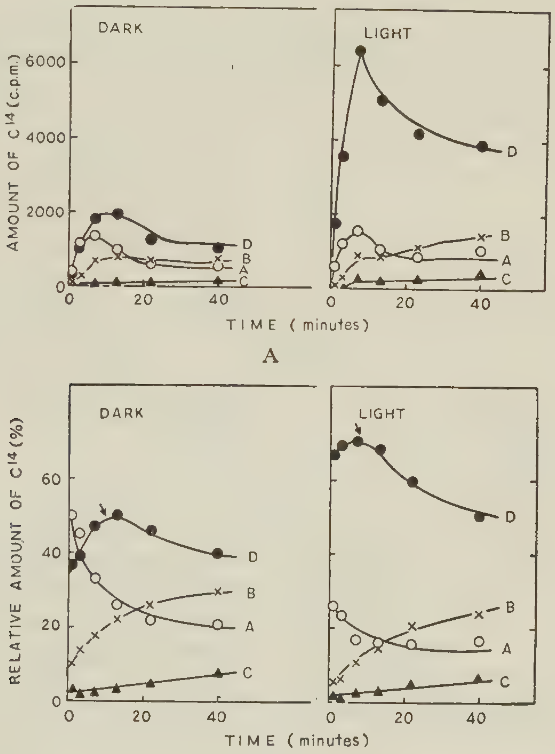
Flow Sheet of Fractionation of Cell Material



acids, amino acids and peptides of low molecular weight; Fraction B is composed mostly of lipids; Fraction C contains most of the nucleic acids besides other undefined substances; and Fraction D contains proteins.

All the experiments were carried out with carboxyl-labeled acetic acid as the substrate.

(1) In one series of experiments, a limited amount of labeled acetic acid was added to the cell suspension, in order to obtain a general picture of the events that took place during the presence, as well as after the exhaustion of the substrate. One of the typical sets of results is shown in Fig. 8A. The data are replotted in Fig. 8 B to show the radioactivity of each fraction in percentage of total incorporation. It is apparent from these results that the incorporation of C^{14} was markedly accelerated by the effect of light. Both in the light and in the dark a peculiar course of C^{14}-



A

B

Fig. 8. Distribution of C^{14} in various fractions of cell material. Reaction vessel: Warburg's manometer vessel; bacterial cell: 31.6 mg. dry weight per vessel; substrate: carboxyl-labeled acetate, 1 μM per vessel; phosphate buffer: pH 7.0, final concentration $M/40$; total volume of reaction mixture: 2 ml.

incorporation was observed with Fraction A (water-soluble). Namely, this fraction contained, at the very beginning of the experiment, a greater portion of the total C^{14} incorporated, which then showed a rapid drop to attain finally a steady low level, while in all the other fractions C^{14} (in percentage of total incorporation) increased more or less markedly. With the exhaustion of the substrate, which is marked with arrows in the figures

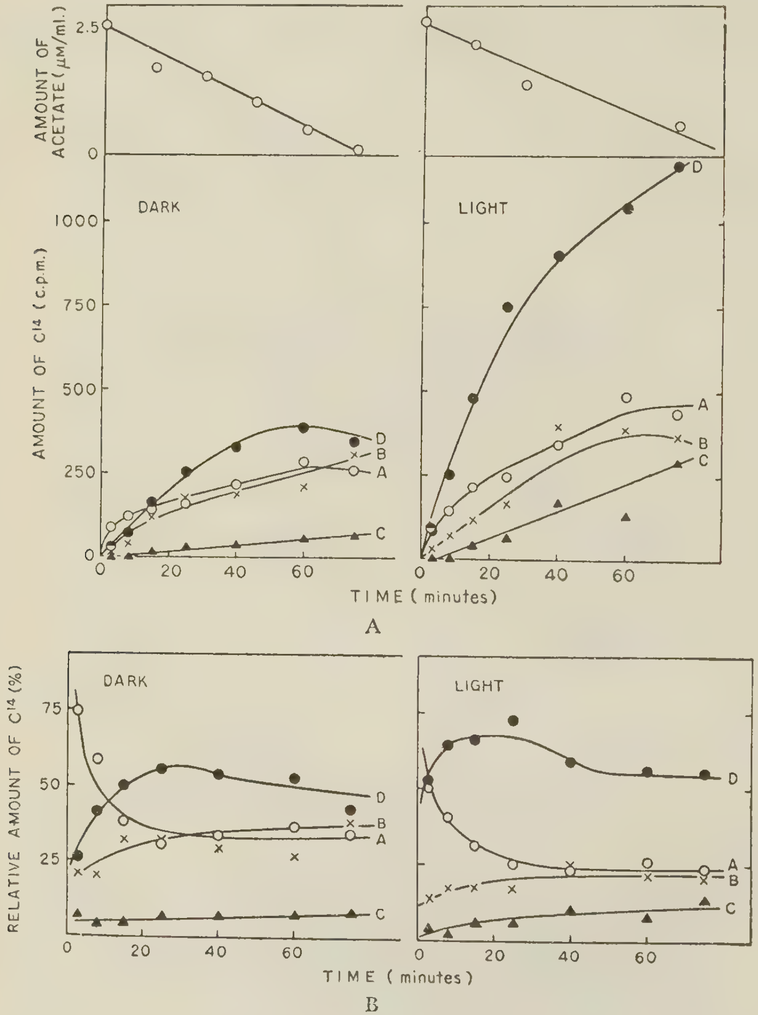


FIG. 9. Distribution of C^{14} in various fraction of cell material.

Reaction vessel shown in Fig. 1 was used; bacterial cells: 91.7 mg. dry weight per vessel; substrate: carboxyl-labeled acetate (4.8 μc/mM), 50 μM per vessel; phosphate buffer: pH 7.0, final concentration $M/40$; initial volume of reaction mixture: 20 ml.

C^{14} in Fraction D was subject to an abrupt drop to assume, within 30 to 40 minutes, a steady level. C^{14} in Fractions B (lipids) and C (nucleic acids), on the other hand, increased, both in absolute amounts and in percentage continuously during the whole reaction period investigated. The increase was slow with Fraction C; the effect of light was also most insignificant with this fraction. The subsequent decrease in the total amount of incorporation as shown in the previous figures (Figs. 6 and 7) might have due mostly to the decrease occuring in Fractions A and D.

(2) The course of C^{14}-assimilation was more closely followed in another set of experiments, in which large amounts of the substrate were added to prolong the time of incorporation. The results are presented in Figs. 9 A, B, which show more clearly the course of events which have been observed in the preceding experiment. At the very beginning of the experiment, C^{14} in Fraction A (water-soluble) formed the largest part of the total C^{14} incorporated, and then it rapidly tapered off with the time of incubation. Fractions B and C showed a gradual increase in percentage of total incorporation. The effect of light was also the same as in the preceding experiment. The efficiency of incorporation was greately elevated by illumination, especially in Fraction D (proteins). Both in the light and in the dark, however, the percentage of C^{14} in this fraction decreased with time, the highest value being observed 20–30 minutes after the beginning of the experiment.

(3) In the last set of experiments, the substrate was added in three succesive lots; (a) 25 μM of non-labeled acetic acid was preliminarily added to set the metabolic system of the cell in working condition. (b) After 45 minutes of incubation, when the bulk of the first addition of acetic acid had been exhausted, 2.5 μM (1.2 μc) of carboxyl-labeled acetic acid was added as the substrate, and the incorporation of C^{14} into various fractions as well as into $C^{14}O_2$ was followed. (c) In order to maintain the cell system at a normal level of metabolism, non-labeled acetic acid (50 μM) was added 10 minutes after the addition of the radioactive substrate, when the latter had been almost exhausted. The results are shown in Figs. 10 A, B and C.

The general aspect of the incorporation and $C^{14}O_2$-production in this experiment is illustrated in Fig. 10 A. At the very beginning of the experiment (*i.e.*, immediately after the addition of the radioactive substrate), the level of total incorporation attained a value as high as 95 per cent and 75 per cent, respectively, of the added amount of substrate, in the light and in the dark. The decrease was rapid in the dark and slow in the light. In this case, the evolution of $C^{14}O_2$ from the radioactive substrate occurred without any lag in time, irrespective of the condition of illumination. As shown in the figure, the evolution of $C^{14}O_2$ was uncomparably high in the dark than in the light. The gradual decline in the rate of output in the light took place at an earlier period of incubation than it was case in the dark experiment. This depression in $C^{14}O_2$-evolution was compensated by

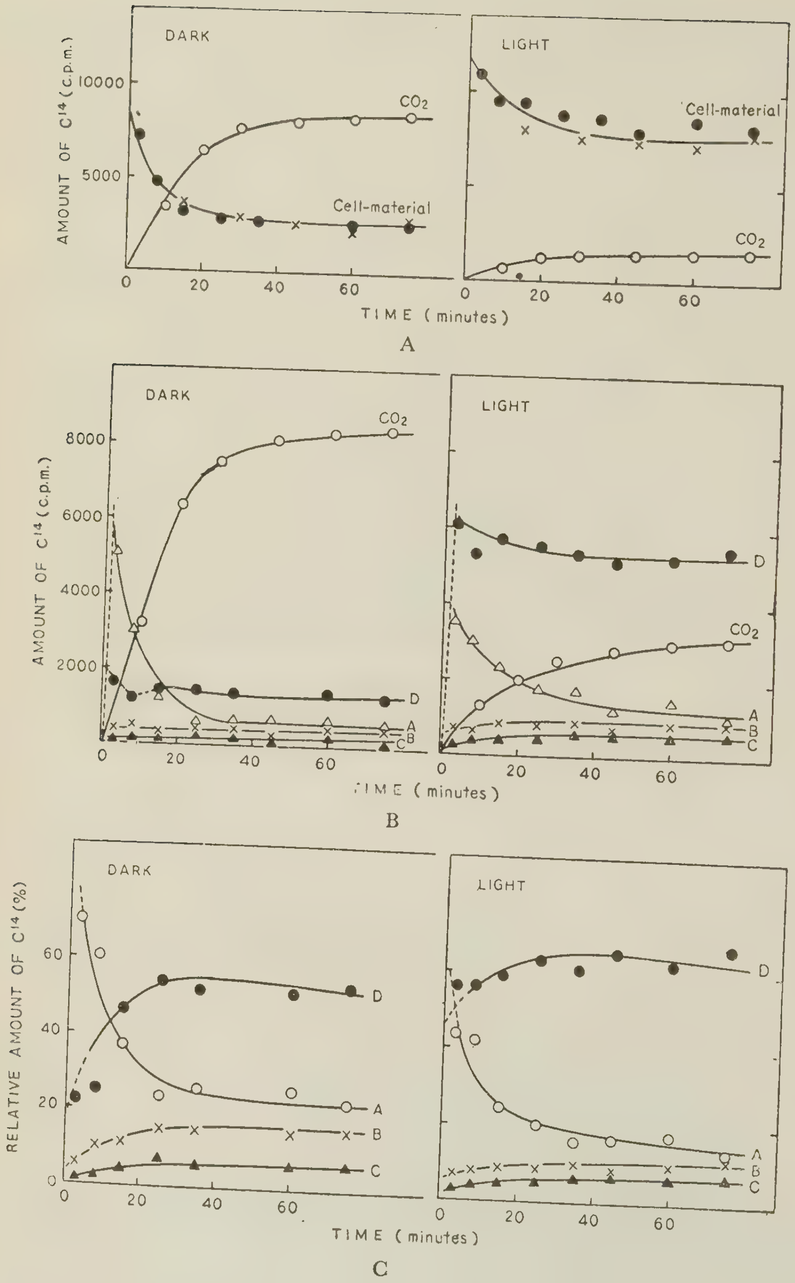


FIG. 10. Distribution of C^{14} in various fraction of cell material.

For the procedure, see text, reaction vessel shown in Fig. 1 was used; bacterial cell: 182 mg. dry weight per vessel; substrate: see text; phosphate buffer: pH 7.0, final concentration *M*/40; initial volume of reaction mixture: 20 ml.

the much elevated yield in the incorporation in the cell material under illumination. The gradual decrease in the rate of $C^{14}O_2$-production at still later stages of incubation (see Fig. 10 A) is evidently due to the limited quantity of the radioactive substrate added in this case.

The results of the fractionation experiments are shown in Figs. 10 B and C. Here again, it was the C^{14} in Fraction A that appeared first in a large quantity and subsequently decreased rapidly. The initial rise in the C^{14}-level in this fraction as well as that in Fraction D (proteins) was almost instantaneous in this case, which was in marked contrast to the more prolonged increse of C^{14} in these fractions observed in the former experiments (see Figs. 8 and 9). In this experiment the increase of C^{14} in Fractions B (lipids) and C (nucleic acids) was relatively slow as compared with that in Fractions A and D. In a period of 10 to 30 minutes, both Fractions B and C showed stationary values of C^{14}-level which were considerably higher in the light than in the dark (Fig. 10 B).

The relative values of C^{14}-level which were finally attained in different fractions after 60 minutes' incubation were as follows;

In the dark:

A (water-soluble): B (lipids): C (nucleic acids): D (proteins): $C^{14}O_2$
=6.6 : 3.7 : 1.7 : 13.1 : 74.8

In the light:

A: B: C: D: $C^{14}O_2$=15.2 : 9.3 : 5.8 : 54.6 : 15.0

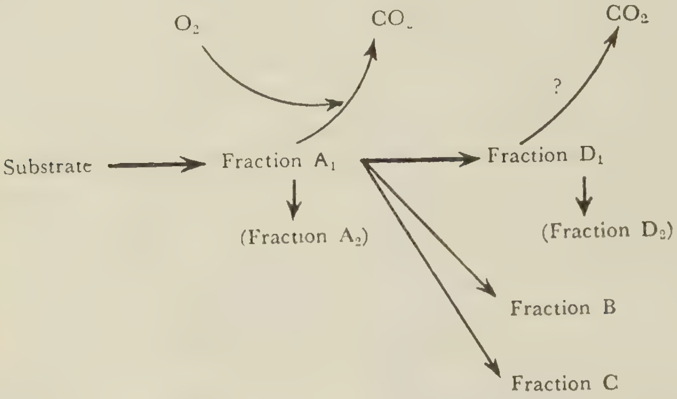
DISCUSSION

All the data presented above seem to warrant the conclusion that the carboxyl-C^{14} of acetate is incorporated most immediately into the substance(s) contained in Fraction A and then into the substance(s) contained in Fraction D. The fact that the C^{14} which had been initially incorporated in Fraction A soon decreased rapidly, while that in other fractions increased more or less markedly, indicates that the initial substance(s) in Fraction A was partly converted to the substances in other fractions and partly evolved as $C^{14}O_2$. The balance of these different ways of conversion was shifted by illumination in favor of the synthesis of cell material, with a result that the evolution of $C^{14}O_2$ decreased and the incorporation of C^{14} in other fractions, especially in Fraction D (proteins), increased. The decrease of C^{14}-level in Fraction D observed at later stages of incorporation (Figs. 8, 9 and 10) may also be due partly to its conversion into $C^{14}O_2$, and partly to its incorporation into other fractions of cell material.

The results of the experiments shown in Fig. 10, indicate that cretain portions of the C^{14} incorporated in Fractions A and D were bound to a relatively stable substance(s) which remained in the same fractions even on prolonged incubation of bacterial cells in the presence of sufficient amounts of substrate. The decrease observed in the C^{14}-level of Fractions A and D may be due to the metabolism of "unstable" portions in these fractions.

The results of the experiment shown in Fig. 10 indicate that the C^{14}-containing substances in Fractions B and C, are relatively stable or interchangeable only in the same respective fractions.

The course of events may most simply be visualized by the following scheme:



where A_1 and A_2, and D_1 and D_2 stand for unstable and stable substances in Fractions A and D, respectively. Fraction A is the water-soluble fraction, Fraction B consists of lipids, Fraction C contains nucleic acids besides unidentified substances and Fraction D is mainly proteins.

The time lag observed in the $C^{14}O_2$-formation from carboxyl-(or methyl-) labeled acetic acid, might have been due to the initial formation of considerable amounts of cell material, especially that in Fraction A and probably partly that in Fraction D. The fact revealed by the experiments shown in Fig. 4, that there was also a similar time lag in the oxygen uptake indicates that the respiratory oxygen uptake is directly connected with the process of $C^{14}O_2$-evolution, (*via* Fraction A). In fact, it has been repeatedly observed by previous investigators, as well as by the present author, that the oxygen uptake, like the CO_2-evolution, by the Athiorhodaceae bacteria was suppressed by illumination. This may be another evidence for the switch mechanism of metabolism caused by illumination, which diverts the pathway of metabolism of the substance(s)—probably contained mainly in Fraction A—from the oxidative to assimilatory one.

In one of the previous papers from this laboratory, Tsukamoto (*9*) reported that the cells of this same organism oxidized acetic acid to succinic and fumaric acids, under dark-aerobic condition. Molecular oxygen was absorbed, however, with no output of carbon dioxide. As a result, the organic acids were found to accumulate in the reaction mixture. The test material used in the present study was found to be capable of metabolizing acetic as well as succinic and fumaric acids. The amount of succinate formed from acetic acid was hardly more than a trace, as revealed by the results of paper chromatographic examination. The discrepancy between the results of Tsukamoto's experiments and ours may most plausibly be due to the difference in the physiological state of the test materials used in the respective studies. Tsukamoto used the cells previously aerated for three days to reduce the endogenous respiration, during which time the cells must have undergone a more or less serious degradation with respect to their physiological activity. In the present study, freshly harvested cells were used throughout the experiments.

In marked contrast to the results obtained by van Niel and others with *Rhodospirillum rubrum*, our organism showed no requirement of "sparking agents"—such as carbon dioxide or C_4-acids—for the metabolism of acetate. There was an induction period of about 10 to 30 minutes in the initial stage of oxygen uptake, (as well as of $C^{14}O_2$-output), which was not abolished by the addition of either fumaric or malic acid. It is the opinion of the present author that his test organism had no requirement for "sparking agent", and that the induction observed was actually caused by the temporary accumulation of certain intermediary metabolic products which were contained probably in Fractions A and D.

In our experments radioactivity appeared, both in the light and in the dark, first in the water-soluble fraction (Fraction A), whereas, Glover, Kamen and Van Gendern (1952) (7), working with *Rhodospirillum rubrum*, reported that, in the light, the radiocarbon of methyl- and carboxyl-labeled acetic acid was most rapidly incorporated in the lipid fraction of cell material. In our experiments, the radioactivity in the lipid fraction (Fraction B) was always far less significant than that in other fractions (A and D). We also failed to observe any difference in the accumulation of radiocarbon in the lipid fraction in the light and in the dark. This disagreement may be due to the difference in the conditions and duration of the reaction in the two cases. Glover *et al.* always performed their experiments with the addition of unlabeled bicarbonate to the reaction mixture, and they examined, in most of their experiments, the earliest stages of 8 to 24 seconds after the addition of the substrate, whereas we investigated the events occurring in considerably longer periods of time.

More detailed investigations on the pathways of methyl- and carboxyl-labeled carbons of acetic acid are being carried out and we hope to report the results in due course.

SUMMARY

1. Aerobic metabolism of acetic acid by an Athiorhodaceae bacterium, *Rhodospseudomonas palustris* was investigated under light and dark conditions.

2. By using labeled acetic acid as the substrate, it was revealed that both the methyl- and carboxyl-carbon of the acid were partially evolved as CO_2 and partially incorporated in cell material. With the two samples of labeled acetate the relative distribution of C^{14} in carbon dioxide and cell material was found to be as follows:

With carboxyl-labeled acetate:

	In cell material	In CO_2
Light	57%	43%
Dark	18%	82%

With methyl-labeled acetate:

	In cell material	In CO_2
Light	88%	12%
Dark	53%	47%

3. The evolution of $C^{14}O_2$ from carboxyl- or methyl-labeled—both in the light and in the dark—showed some induction period, whereas the incorporation of C^{14} into cell material occurred without any lag of time.

4. By fractionating the cell material into four portions (water-soluble, lipids, nucleic acids, proteins), the relative rates of C^{14} incorporation from carboxyl-labeled acetate were investigated, and the results obtained indicated that, both in the light and in the dark, the C^{14} was first incorporated in the water-soluble fraction, from which it subsequently went partially into CO_2 and partially into other fractions, especially rapidly and in a large quantity into the proteinous fraction. The balance between the oxidative (CO_2-evolving) and the assimilative metabolism was markedly shifted by illumination in favor of the synthesis of cell material, with a result that the evolution of $C^{14}O_2$ decreased and the translocation of C^{14} from the water-soluble fraction into other fractions (especially into the proteinous fraction) increased.

It is a pleasure for the author to express his thanks to Prof. H. Tamiya for his continuous guidance and keen interest throughout this work. His thanks are also due to Prof. A. Takamiya for helpful advice and kind suggestions. The present work was supported by a grant from the Ministry of Education, which is gratefully acknowledged here.

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The Journal of Biochemistry, Vol. 45, No. 9, 1958

STUDIES ON EGG YOLK PROTEINS

IV. ELECTROPHORETIC COMPOSITION OF EGG YOLK PROTEINS

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(Received for publication, September 24, 1957)

In the previous works (*1*, *2*), the author has found a suitable condition for electrophoretic studies of the yolk proteins and obtained some evidences concerning the inhomogeneities and mutual relations of several preparations isolated hitherto (phosvitin of Mecham and Olcott (*3*), lipovitellin of Alderton and Fevold (*4*), and lipovitellenin of Fevold and Lausten (*5*)), and, subsequently, two major lipoproteins, α- and β-lipovitellin, have been isolated from the egg yolk in the electrophoretical pure form (*6*).

In the present paper, the protein composition of the egg yolk has been determined on the basis of the results of these electrophoretic studies. The lipid and phosphorus contents expected from this protein composition have been compared with the values obtained experimentally. In addition, some informations have been obtained concerning the phosphorus distribution in the yolk proteins.

EXPERIMENTAL

Materials, analytical methods, and electrophoretic experiments have been performed as before (*1*, *2*).

RESULTS

At present, nine components are recognized in the egg yolk from the results of electrophoretic studies; namely, phosvitin, α- and β-lipovitellin, three livetin components (L_1, L_2, and L_3), and three minor components (including γ-component) (*1*, *2*, *6*). However, it is unable to obtain the percentages of these components in the egg yolk from the electrophoretic pattern of the total yolk protein, because the peaks of livetin components overlap with those of α- and β-lipovitellin and the separation of the peaks of α- and β-lipovitellin is insufficient to estimate the percentages. Therefore, the electrophoretic composition of the egg yolk proteins has been determined according to the method as stated in the subsequent sections.

Phosvitin—In the yolk proteins, phosvitin was the most rapid moving component and separated completely from the other components on electrophoretic analysis (*1*, *2*). Therefore, its percentage in the total yolk protein could be computed easily from the electrophoretic patttern of the total yolk protein. The percentage of phosvitin in the fraction*, which was obtained by removing the livetin fraction from the total yolk protein by the complete water dialysis, was determined from the electrophoretic pattern of that fraction. The results are shown in Table I.

Livetin—It is known that the livetin fraction consists of three electrophoretic components (L_1, L_2, and L_3) (*1, 7*). The quantity of protein included in the livetin fraction was able to be obtained by a simple calculation using the percentages of phosvitin in the total yolk protein and the WDP fraction. The results are shown in Table I.

TABLE I

Percentages of Phosvitin and Livetin Fraction in the WDP Fraction and Total Yolk Protein

Exp. no.	pH	μ	Prot. conc.	Phosvitin in the WDP fraction	Phosvitin in the total yolk prot.	Livetin fraction in the total yolk prot.
1	9.8	0.2	2.0%	9.4%	10.8%	13.0%
2	9.8	0.2	2.2	8.8	10.3	14.6
3	9.8	0.2	2.0	7.5	9.0	16.7
4	9.1	0.2	2.1	9.0	10.3	12.6
5	10.4	0.2	2.0	7.8	9.2	15.2
Average				8.5	9.9	14.2

α- and β-Lipovitellin—As stated above, it was difficult to obtain the percentages of these components from the electrophoretic pattern of the total yolk protein or the WDP fraction. However, in the precipitate fraction (Insoluble A in Scheme 1 in (*6*)) obtained at 0.03 *M* magnesium sulfate in the first dilution step of the preparation method of α- and β-lipovitellin (*6*), the separation of the peaks of α- and β-lipovitellin was comparatively good. The electrophoretic composition of this fraction was reported in (*6*); namely, phosvitin 19.6, α-lipovitellin 38.5, β-lipovitellin 19.3, and the others 22.6 per cent. And also, the electrophoretic composition of the fraction, which was obtained by removing the livetin fraction from the filtrate (Soluble B in Scheme 1 in (*6*)) obtained at 0.03 *M* magnesium sulfate, was as follows; α-lipovitellin 8.6, β-lipovitellin 82.2, and the others 9.2 per cent. Therefore, the percentages of α- and β-lipovitellin in the WDP fraction could be calculated by estimating the quantities of the Insoluble A and fraction which was obtained by removing the livetin fraction from the Soluble B. Since the percentages of phosvitin in the Insoluble A and WDP fraction are 19.6 and 9.9 per cent, respectively,

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* In the following sections, this fraction was called "WDP fraction" for simplification.

$$\frac{19.6\times x}{x+y}=9.9 \qquad \therefore \quad \frac{x}{y}=1.02$$

where x and y are the quantities of the Insoluble A and the fraction which was obtained by removing the livetin fraction from the Soluble B, respectively. Thus, the percentages of α- and β-lipovitellin in the WDP fraction could be obtained as follows:

$$\alpha\text{-lipovitellin}\,;\ (38.5\%\times 1.02+8.6\%\times 1)/2.02=23.7\%$$
$$\beta\text{-lipovitellin}\,;\ (19.3\%\times 1.02+82.2\%\times 1)/2.02=50.4\%$$

These values could be converted to those in the total yolk protein, since the percentages of livetin fraction in the total yolk protein was already known. The obtained results are shown in Table II.

Other Components—In addition to phosvitin, α- and β-lipovitellin, three minor components could be recognized electrophoretically in the WDP fraction. Two of these moved between phosvitin and α-lipovitellin. The remaining one (γ-component in (*6*)) followed β-lipovitellin. The percentage of these components in the WDP fraction was obtained as the above:

$$(22.6\%\times 1.02+9.2\%\times 1)/2.02=16.0\%$$

Since the percentage of γ-component in the fraction obtained by removing the livetin fraction from the Soluble B was 7.0 per cent (*6*), the percentage of it in the WDP fraction became to 3.5 per cent and that of the other two components to 12.5 per cent. On the fractionation, γ-component acted with β-lipovitellin and the other two with phosvitin (*6*). It was confirmed from the lipid measurement that the latter has no alcohol-extractable lipid. The γ-component was presumed to be a lipoprotein (*6*). It was presumed from the phosphorus measurement that the phosphorus content of the other two components was similar at the most with that of α-lipovitellin. The contributions of these minor components to the phosphorus and lipid contents of the total yolk protein will be small, since the percentage of these components in the total yolk protein is low.

TABLE II

Protein Composition of WDP Fraction and Total Yolk Protein by Electrophoretic Analysis

	Phosvitin	Others	α-Lipovitellin	β-Lipovitellin	γ-Component	Livetin (L_1 L_2 L_3)
WDP fraction	9.9%	12.5%	23.7%	50.4%	3.5%	0%
Total yolk protein	8.5	10.8	20.3	43.2	3.0	$\underbrace{2.8\ \ 8.2\ \ 3.2}_{14.2}$

The Alcohol-Extractable Lipid Content of WDP Fraction—When the yolk was diluted with two volumes of 10 per cent sodium chloride and extracted with ether until the ether layer became colorless, the obtained WDP fraction represented an alcohol-extractable lipid content of 27 to 29 per cent. Even though the extraction was repeated further 10 times by three volumes of ether, this lipid content was constant within the experimental error. However, when the WDP fraction was redispersed in a small amount of water and continued extracting with ether, the lipid content decreased to 19 to 21 per cent. And, this lipid content was also unchanged even after further 10 times of ether extration.

The phosphorus Content of WDP Fraction—After dispersing in water and extracting with ether, the WDP fraction represented a phosphorus content of 2.0 to 2.1 per cent. The phosphorus content of WDP fraction having the lipid content of 27 to 29 per cent was 0.1 to 0.2 per cent higher than the above. After an alcohol extraction, the phosphorus content decreased to 1.5 to 1.6 per cent.

DISCUSSION

The bound lipid has usually been defined as that which is not extractable by cold ether. However, this criterion has no theoretical ground. As shown above, a considerable amount of the lipid of WDP fraction which was not extractable in 10 per cent sodium chloride solution was able to be extracted in the state dispersed in water. These lipid molecules are considered to be protected somehow in such way that they are not removed by ether in the salt solution. The same result could be observed also in respect to β-lipovitellin. The β-lipovitellin represented a lipid content of 37 to 39 per cent when it was extracted with ether at 0.03 M of magnesium sulfate (*6*). However, when it was redispersed in a small amount of water and continued extracting with ether, the lipid content decreased to 35 to 36 per cent. And also, it could be estimated that when β-lipovitellin was extracted in 10 per cent sodium chloride solution, it showed the lipid content of 42 to 45 per cent*.

The depression of the lipid content of WDP fraction as stated above could not be explained quantitatively by only that of β-lipovitellin. It seems that this difference is due to the similar lipid depression of α-lipovitellin. Recently, Vandegaer, Reichmann, and Cook (*8*) have isolated a lipoprotein from the egg yolk by means of ultracentrifugation, which contained about 18 per cent lipid and about half of it could be extracted with ether**. This fact shows that in α-lipovitellin there exists some amount of weaker bound lipid than the alcohol-extractable one. It is important to know how these ether-extractable lipids influence the physico-chemical properties of lipoproteins. These problems have to be dissolved hereafter in relation to the structure of lipoprotein.

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* This was estimated as follows. To the filtrate obtained at 0.03 M of magnesium sulfate (Soluble B in Scheme 1 in (*6*)) was added solid sodium chloride till 10 per cent and the mixture was then extracted with ether until ether layer became colorless. And, it was separated from the livetin fraction by dialyzing against water. The above value of lipid content was estimated from the lipid content and electrophoretic composition of this fraction.

** It is considered from the lipid content and yield that this lipoprotein corresponds to α-lipovitellin.

The lipid and phosphorus contents of WDP fraction could be computed by the protein composition of WDP fraction and the lipid and phosphorus contents of the protein components. In estimating the protein composition of WDP fraction, the fractions used in the electrophoretic analysis were all extracted with ether in 10 per cent sodium chloride solution. Therefore, the value of 43.5 per cent was used as the lipid content of β-lipovitellin. For the lipid content of α-lipovitellin, the value of 18 per cent was used (*8*), since it was not obtained here experimentally.

$$(18\% \times 23.7 + 43.5\% \times 50.4)/100 = 26{\cdot}2\%$$

Assuming that γ-component is a lipoprotein having a lipid content similar with β-lipovitellin, this value increases to 27.7 per cent. Since the percentages of α- and β-lipovitellin increase more or less by considering their low refractive index increments, this value will increasd further a little more. However, in estimating the lipid content of WDP fraction extracted with ether in the state dispersed in water, 6.5 and 35.5 per cent must be used as the lipid contents of α- and β-lipovitellin, respectively. In this case, the percentages of α- and β-lipovitellin in the protein composition of WDP fraction somewhat decrease, but at the same time they increase a little by considering their low refractive index increments. Accordingly, the lipid content of WDP fraction extracted in the state dispersed in water can be computed approximately by using the same percentages of α- and β-lipovitellin.

$$(6.5\% \times 23.7 + 35.5\% \times 50.4)/100 = 19.4\%$$

If γ-component is added as the above, this increases to 20.7 per cent. Thus, these are in favorable agreement with the values obtained experimentally.

The phosphorus content of WDP fraction can be estimated also as follows by using the phosphorus contents of each component reported in the previous work (*6*).

$$(9.7\% \times 9.9 + 0.7\% \times 23.7 + 1.6\% \times 50.4)/100 = 1.92\%$$

If the minor components are added, this value will increase a little more. The protein composition of WDP fraction after an alcohol extraction was computed as follows; phosvitin 12.5, α-lipovitellin 27.9, β-lipovitellin 41.0, γ-components 2.8, and the others 15.8 per cent (assuming that the lipid contents of α- and β-lipovitellin are 6.5 and 35.5 per cent, respectively). Therefore, the phosphorus content of WDP fraction after an alcohol extraction can be estimated by using the phosphorus contents of α- and β-vitellin reported in the previous work (*6*).

$$(9.7\% \times 12.5 + 0.6\% \times 27.9 + 0.3\% \times 41.0)/100 = 1.50\%$$

Thus, these are also in good agreement with the values obtained experimentally.

By the similar calculation*, some informations could be obtained concerning the phosphorus distribution in the yolk proteins. In the WDP fraction containing 27 to 29 per cent lipid, 47 per cent of the total phosphorus was the lipid phosphorus and the remaining was the protein phosphorus. And, 78 per cent of the lipid phosphorus was attributed to β-lipovitellin and 82 per cent of the protein phosphorus to phosvitin. But, in the WDP fraction containing 19 to 21 per cent lipid, 36 per cent of the total phosphorus was the lipid phosphorus and 89 per cent of it was attributed to β-lipovitellin. The protein phosphorus was 64 per cent of the total phosphorus and 73 per cent of it was attributed to phosvitin. Thus 74 to 84 per cent of the total phosphorus of egg yolk proteins belongs to both of phosvitin and β-lipovitellin. Phosvitin contains the phosphorus as the protein phosphorus and β-lipovitellin about 90 per cent of it as the lidid phosphorus. The detailed distribution of the phosphorus in the egg yolk proteins is shown in Fig. 1. Recently, Clegg, Hein, Suelter, and McFarland (*10*) have reported on the distribution of radioactive phosphorus in the electrophoretic components of egg yolk proteins. Although the electrophoretic conditions employed by them were rather different from those in this work, both distribution patterns of the phosphorus showed a considerable similarity.

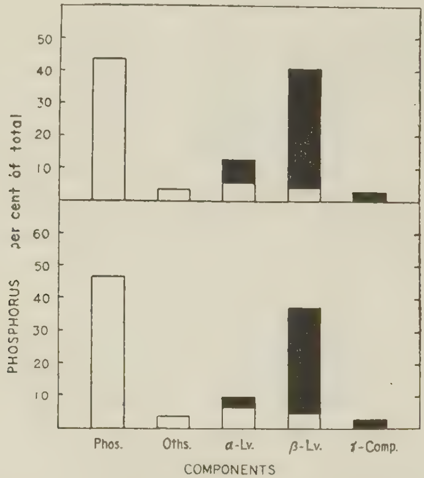

Fig. 1. Phosphorus distribution in the yolk proteins*. Upper, the distribution in the WDP fraction containing 27 to 29 per cent lipid; lower, the distribution in the WDP fraction containing 19 to 21 per cent lipid: white, protein-phosphorus; black, lipid-phosphorus.

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* A value of 3.7 per cent was used as the phosphorus content of phospholipid (*5*). The contribution of livetin fraction to the total phosphorus of egg yolk proteins was neglected in the present calculation, since the phosphorus content of livetin fraction was very low (0.05 per cent (*9*)).

Accordingly it seems that the distribution of radioactive phosphorus reflects approximately the distribution of total phosphorus.

The WDP fraction is essentially equal to the old lipovitellin prepared by the dialysis or dilution method (*11*, *12*). The principal causes, for which the obtained preparations do not give a constant lipid or phosphorus content, are considered to exist in the procedures of ether extraction and water dialysis*. The water dialysis for a insufficient time or a high water temperature causes some loss of β-lipovitellin. For example, in a case the dialysis was carried out at 20°, the livetin fraction which was obtained by boiling the supernatant represented a lipid content of about 20 per cent. Namely, in this case, an almost equal amount of β-lipovitellin with the livetin fraction remained in the supernatant**.

When the dialysis was repeated several times, we must pay attention also to the loss of phosvitin. This is suggested by the solubility properties of phosvitin (*2*). A small loss of phosvitin would not be apparent in terms of the total protein, but corresponds to a considerable fraction of the phosphorus. Loss of phosvitin in this procedure was demonstrated by the experiments of Francis (*13*) in which P^{32}-labeled phosvitin was mixed with lipovitellin and the mixture was precipitated by dilution. On each successive precipitation phosvitin was lost from the lipovitellin as shown by radioactivity determinations. Similarly, the lipovitellin prepared by Alderton and Fevold (*4*) without precipitation by dialysis contained 2.3 per cent phosphorus, whereas after three precipitations it decreased to 1.5 per cent.

SUMMARY

1. From the results of the electrophoretic studies of the egg yolk and the fractions, the protein composition of the egg yolk has been determined.

2. It has been shown that the lipid and phosphorus contents of the total yolk protein are in good agreement with the values estimated on the basis of the lipid and phosphorus contents of the protein components.

3. The phosphorus distribution in the yolk proteins has been made clear by the similar calculations.

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* The influences due to the differences of lineages, feeds, and seasons are disregarded.

** Since β-lipovitellin precipitates completely with half saturation of ammonium sulfate, this will become a cause for the high phosphorus content of livetin fraction.

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The Journal of Biochemistry, Vol. 45, No. 9, 1958

STUDIES ON THE CHEMICAL PROPERTIES OF THE LIGNIN OF BAMBOO-STALK

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(Received for publication, September 3, 1957)

Relatively few investigations have hitherto been made on the lignins of monocotyledones, especially informations about the lignin of bamboos are very, meagre. Hibbert *et al.* studied the structural differences of the lignin of bamboo-shoot from that of coniferous and deciduous trees by alkaline nitrobenzene oxidation (*1*, *2*) and ethanolysis (*3*). They obtained vanillin, syringic aldehyde and *p*-hydroxybenzaldehyde as oxidation products by the former method, whereas α-ethoxypropiovanillone and vanillin were identified by the latter method.

The present author has been able to obtain some other products as well as the compounds identified by Hibbert *et al.* upon alkaline nitrobenzene oxidation, ethanolysis and hydrolysis of the bamboo lignin. The results are described in the present paper.

EXPERIMENTAL

A freshly cut bamboo-stalk (Phyllostachys edulis), 9.28 m. in height and 13.2 cm. in diameter, was pulverized to 40-60 mesh following conventional methods, and it was extracted successively with ethanol-benzene (1 : 1 by volume) for 24 hours, with ethanol for 24 hours, and with warm water until the extract was colorless. The extracted meal was air-dried and used as the starting material (lignin content, 261 per cent).

Alkaline Nitrobenzene Oxidation—By the method of Creighton *et al.* (*1*), 30 g. of material was oxidized with alkaline nitrobenzene and the alkaline reaction mixture was steam distilled to remove nitrogeneous products and was filtered. The filtrate was acidified, and continuously extracted for 70 hours with ether. The ether extract was shaken successively with sodium bisulfite (20 per cent), sodium bicarbonate (8 per cent) and sodium hydroxide (5 per cent).

Examination of the Bisulfite-Soluble Fraction—The aqueous bisulfite solution was acidified, the sulfur dioxide removed under reduced pressure and diluted to 400 ml. The total aldehydes in an aliquot (40 ml.) were determined by precipitation as the 2,4-dinitrophenylhydrazones. Total bisulfite-soluble oils were isolated by extraction of the remaining solution with ether, and springic aldehyde, vanillin and *p*-hydroxybenzaldehyde were isolated from the oils using the method of Creighton *et al.* (*4*), syringic aldehyde was precipitated as its ammonium salt with anhydrous ethanol-ammonia, vanillin was then precipitated with anhydrous ether-ammonia from the filtrate, and finally *p*-hydroxybenzaldehyde was obtained from the final filtrate. In blank experiments with syringic aldehyde, vanillin, and *p*-hydroxybenzaldehyde carried out under identical conditions, syringic aldehyde was precipitated as syringyl ammonium salt with a recovery of 90–95 per cent, and vanillin was obtained with a recovery of 80-90 per cent. *p*-Hydroxybenzaldehyde which was not precipitated by both anhydrous ethanol-ammonia and anhydrous ether-ammonia was determined as its 2,4-dinitrophenylhydrazone with a recovery of 92–95 per cent. Syringic ammonium fractionated as above, was hydrolyzed with dilute H_2SO_4 and extracted with ether. Syringic aldehyde was obtained as needle shaped crystals after evaporation of the ether; m.p. 109–110°. Semicarbazone and 2,4-dinitrophenylhydrazone were prepared by ordinary methods. These melted at 183–185° and 232–235°, respectively, and when mixed with authentic speciments, no depression of the melting points was detected. Crystals of vanillin and *p*-hydroxybenzaldehyde were similarly obtained from vanillin ammonium and the concentrate of the final filtrate, respectively. The melting points; vanillin, 81-82°; semicarbazone, 231-232°; 2,4-dinitrophenylhydrazone, 260–262°; *p*-hydroxybenzaldehyde, 115–116°; semicarbozone, 222–223°; 2,4-dinitrophenylhydrazone, 275-277°. These melting points were not depressed when mixed with authentic speciments. Paperchromatography was also employed in order to acquire additional evidence for the identity. The ethanolic solutions of samples were spotted on papers and ascending two dimensional paper

TABLE I

R_f Values and Color Reactions of Compounds Obtained by Nitrobenzene Oxidation of Bamboo-Stalk

Compound	R_f value			Color reaction	
				Diazotized sulfanilic acid	2,4-Dinitro phenylhydrazine-HCl
Syringic aldehyde	0.30[1]	0.66[2]	0.68[3]	Reddish-pink	Dark-orange
Vanillin	0.38	0.71	0.78	Orange	Orange
p-Hydroxybenzaldehyde	0.43	0.84	0.85	Yellow	Dark-orange
Syringic acid	0.05	0.82	0.18	Reddish-pink	Colorless
Vanillic acid	0.07	0.87	0.21	Orange	Colorless
p-Hydroxybenzoic acid	0.12	0.89	0.37	Yellow	Colorless
Unknown	0.95	0.02	—	Pale orange	Light orange

1) *n*-Butanol, saturated with 3 per cent NH_4OH.

2) Aqueous layer of a mixture of benzene, ligroin, water and methanol (50:50:50 1 by volume.)

3) Water-saturated *n*-butanol.

chromatography was mainly employed using the organic layers from a mixtures of benzene, ligroin, methanol, water (50 : 50 : 1 : 50 by volume) (*5*), and *n*-butanol saturated with 3 per cent NH_4OH as solvents. After spraying each with diazotized sulfanilic acid and 2,4-dinitrophenylhydrazine-HCl, respectively, the R_f values and color reactions were compared with those of authentic specimens. The results are shown in Table 1.

Examination of Sodium Bicarbonate- and Sodium Hydroxide Extracts—These extracts were acidified, extracted with ether and the solvents removed. Identification by paper chromatography was made using the same solvent as employed above, and diazotized sulfanilic acid as the spraying reagent. Syringic acid, vanillic acid, and *p*-hydroxybenzoic acid were identified in both fraction by comparison of R_f values and color reactions of the sample with those of authentic specimens (Table I).

The results of semiquantitative determinations of each fraction, and of each aldehyde in the bisulfite-soluble fraction obtained as described above are given in Table II. As seen in the Table, the content of total aldehydes calculated as percentage of 70 per cent H_2SO_4 lignin, and the ratio of syringic aldehyde and vanillin to *p*-hydroxybenzaldehyde are roughly in agreement to those reported by Hibbert *et al.* (*1*). Hibbert *et al.* found that the quantity of total aldehydes obtained from the lignins of monocotyledones amounted to 20–35 per cent and that the ratio of vanillin to syringic aldehyde were 1 : 1–1 : 3, although there were some variations with different plant species.

TABLE II

Fractionation of Nitrobenzene Oxidation Products of Bamboo-Stalk

Total aldehydes* 26.5%		$NaHCO_3$-soluble* 0.8%	NaOH-soluble* 3.5%
Syringic* aldehyde	Vanillin*	*p*-Hydroxybenzaldehyde*	
12.5%	8.5%	5.5%	
(1.6	1.3	1)	

* Based on weight of initial Klason lignin.
Figures in parenthesis show ratio of molecular weight.

Ethanolysis (*6*)—The dry material (464 g.) was refluxed in CO_2 atmosphere with 4 liters of 99 per cent ethanol containing 80 g. anhydrous hydrogen chloride for 48 hours. The alcoholic extract was neutralized with $NaHCO_3$, filtered and concentrated to about 700 ml. under CO_2. The concentrated alcoholic solution was poured into water (A) (6 liters) with vigorous stirring. The ethanol lignin which separated was filtered by suction. The lignin was dissolved in acetone (500 ml.) and the solution was poured into water (B) (4 liters) with continuous stirring to remove the adsorbed oils. The resulting ethanol lignin was air-dried and extracted with benzene (C) for 24 hrs. using a Soxhlet extractor. The combined aqueous liquors (A+B) containing the ethanolysis oils were concentrated to about 700 ml. under reduced pressure (50°, bath temperature) and after acidifying with dilute H_2SO_4, they were extracted with benzene (D) continuously for 100 hrs. The combined benzene extract (C+D) were dried and the solvent removed *in vacuo*. The obtained crude ethanolysis oils (67.1 g.) were dissolved in acetone (600 ml.) and the solution poured into petroleum ether with vigorous stirring to separate the petroleum ether soluble fraction (40.8 g.) from petroleum ether insoluble fraction (24.5 g.). The soluble fraction was dissolved in benzene (300 ml.) and the solution was extraced successively with 20 per cent $NaHSO_3$, 8 per cent $NaHCO_3$ and 5 per cent NaOH. Each of the extracts was treated as described below according to Hibbert *et al.*, and throughout the examinations of the fractions, paper chromatography as well as other analytical methods were used for the identification; the same solvents and spraying regents employed in the nitrobenzene oxidation were mainly used for paper chromatography.

TABLE III

Yields of Ethanolysis Products Isrlated from Bamboo-Stalk

Product	Weight (*g.*)	Klason lignin		Per cent of initial Klason lignin
		(%)	(*g.*)	
Starting material bamboo-meal	464	26.1	121.1	100
Residual meal	241	14.7	35.4	29.2
Ethanol lignin	54.2			44.8
Crude water soluble oils	67.2			55.5
Total recovery of initial Klason lignin				129.5

No correction was made for introduced ethoxy group on the ethanolysis.

The Bisulfite Soluble Compounds—*a*) *Diketones*—Bisulfite soluble fraction (7.5 g.) was dissolved in water (2 liter) and the solution was treated with hydroxylamine sulfate, nickel chloride and sodium acetate as described by Kulka *et al.* (*7*). The precipitated nickel glyoxime salts were collected by centrifugation, dried at 100°. The salts were decomposed with 12 *N* H_2SO_4 and the diketones were extracted with benzene (weight 2.1 g.). Syringoyl methyl ketone was separated from vanilloyl methyl ketone by precipitating the former from ethanol solution as its ammonium salt. The ammonium salt of syringoyl methyl ketone was decomposed with an equivalent amount of dilute H_2SO_4. The free ketone separated out in the form of yellow needles on standing in refrigerator, and these were recrystallized from ligroin; m.p. 80-81°. Monosemicarbazone and quinoxaline derivatives were prepared and the melting points were 209–210°, and 160–161°, respectively; these melting points were in good agreement with those reported by Kulka *et al.* On the other hand, the solvent of the filtrate after separating the ammonium salt of syringoyl methyl ketone, was removed immediately and the residue was dissolved in dry ether (50 ml.). Vanilloyl methyl ketone was then precipitated as its ammonium salt with dry ammonia. The salt was hydrolyzed with dilute H_2SO_4 and extracted with ether. On evaparation of the ether, vanilloyl methyl ketone separated out as a yellow oil which crystallized on standing. The crystal, after one crystallization from ligroin, melted at 68–69° and gave no depression when mixed with an authentic sample of vanilloyl methyl ketone. The quinoxaline prepared from this product melted at 161–162° and showed no depression when mixed with a pure synthetic product.

TABLE IV

Fractionation of Crude Water-Soluble Oils from Bamboo-Stalk

Fraction	Weight (*g.*)	Percent of starting material	Per cent of initial Klason lignin
Staring material	67.2	100	55.5
Petroleum ether-insoluble	24.5	36.5	20.2
Petroleum ether-soluble	40.8	60.7(100)	32.7
Bisulfite-soluble	7.5	11.2(18.4)	6.2
Bicarbonate-soluble	0.7	1.0(1.7)	0.6
Alkali-soluble	23.5	35.0(57.7)	19.4
Neutral	6.5	9.7(15.9)	5.4

b) *Aldehydes*—The fitrate after removing the nikel salts of the diketones was extracted with benzene, and the mixture of aldehydes obtained by bisulfite extraction of the benzene extract was treated as described in the section of the nitrobenzene oxidation. Vanillin, syringic aldehyde and *p*-hydroxybenzaldehyde were identified by means of paper chromatography as well as by melting points and mixed melting points. Further, a faint spot corresponding to that of 1-(4-hydroxy-3.5-dimethoxyphenyl)-2-propanone was detected by paper chromatography in the fraction.

c) *Oximes* (*8*)—The benzene solution containing the oximes was dried and the solvent was removed. The residue was hydrolyzed with 7 *N* H_2SO, and the resulting free carbonyl compounds were examined by paper chromotography. Distinct spots corresponding to those of vanillin, syringic aldehyde, 1-(4-hydroxy-3-methoxyphenyl)-2-propanone, and 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-propanone, as well as a faint spot of 2-hydroxy-(4-hydroxy-3-methoxyphenyl)-1-propanone were obtained.

The Alkali-Soluble Fraction (*9*)—The alkali-soluble ethanolysis oils (23.5 g.)

were separated into ethanol-ammonia insoluble, ether-ammonia insoluble, and ether-ammonia soluble fractions, respectively, after Kulka *et al.* The ammonium salts and the ammonia-ether soluble fraction were separately dissolved in water, and the solutions were acidified and extracted with benzene. After removing the solvents three oily fractions were obtained, an ethanol-ammonia insoluble fraction (5.5 g.), an ether-ammonia insoluble fraction (10.9 g.) and an ether-ammonia soluble fraction (6.1 g.).

a) *Investigation of the Ethanol-Ammonia Insoluble Fraction*—This was a brown viscous oil, and a part of the substances crystallized on standing in a refrigerator. This fraction gave ten spots corresponding to the compounds shown in Table V. Another part (3 g.) of the fraction was distilled at a pressure of 1.0 mm. Hg and a viscous yellow fraction (b.p. 165–180°, 0.5 g.) was obtained. The distillate gave a distinct spot corresponding to that of 2-ethoxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone, and the fraction was then methylated with diazomethane (*10*). The methylated compound, after repeated crystallization from petroleum ether, melted at 78–80°. *Analysis*, Calcd. for $C_3H_7O(OCH_3)_2OC_2H_5$: alcohoxyl as methoxyl 39.0, Found: 38.9. Thus, the compound is the methyl ether of 2-ethoxy-1-(4-hydroxy-3-methoxy-

TABLE V

R_f Values of Compounds Obtained from Ethanolysis Oils of Bamboo-Stalk

Compound	R_f value		NaHSO$_3$-soluble fraction		NaOH-soluble fraction	
R′-COOH	0.05[1]	0.0[2]	+		+	
R-COOH	0.07	0.0	+		+	
R′-CHO	0.28	0.37	+++		−	
R-CHO	0.40	0.48	+++		−	
R″-CHO	0.52	0.01	+		−	
R′-CO·CO-CH_3	0.43	0.57	+++		+	
R-CO·CO-CH_3	0.53	0.67	+++		+	
R′-CH_2·CO-CH_3	0.73	0.50	++		−	
R-CH_2·CO-CH_3	0.87	0.62	++		−	
R-CO·CHOH-CH_3	0.34	0.17	+		+	
R′-CO·CH(OEt)-CH_3	0.80	0.79	−		+++	
R-CO·CH(OEt)-CH_3	0.85	0.88	−		+++	
R-CH(OEt)·CO-CH_3	0.90	0.91	−		+++	
Unknown			0.48[1]	—	0.77	—
Unknown			—	0.59	0.69	—
Unknown					—	0.07
Unknown					—	0.27

1) *n*-Butanol saturated with 3 per cent NH_4OH.

2) Organic layer of a mixture of water, ligroin, chloroform and methanol (5 : 7 : 2 : 1 by volume). R : guaiacyl, R′ : syringyl, R″ : *p*-hydroxyphenyl.

phenyl)-1-propanone (According to Cramer *et al.*, the methyl ether has m.p. 81–82°). On the other hand, the undistilled residues were dissolved in dry pyridine and treated with *p*-nitrobenzoyl chloride (*11*). The reaction product melted at 141–142° after repeated recrystallizations from ethanol. *Analysis*, Calcd. for $C_{20}H_{21}NO_8$: alcohoxyl as methoxyl 23.1, Found: 23.0. Thus, the compound was the *p*-nitrobenzoate of 2-ethoxy-1-(4-hydroxy-3.5-dimethoxyphenyl)-1-propanone (The melting point of the *p*-nitrobenzoate is 140–142° according to Hunter *et al.*).

b) *The Ether-Ammonia Insoluble Fraction*—This fraction gave the same 10 spots as found on the chromatogram of the preceeding fraction, but the spot of 2-ethoxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone was very distinct compared to other spots. The methyl ether of 1-ethoxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone was obtained by the same procedure as mentioned above.

c) *The Ether-Ammonia Soluble Fraction*—Eight spots including 1-ethoxy-1-(4-hydroxy-3-methoxyphenyl)-2-propanone and 2-ethoxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone were found on the paper chromatograms and the spot of 1-ethoxy-1-(4-hydroxy-3-methoxyphenyl)-2-propanone was very distinct compared to that of 2-ethoxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone.

The Sodium Bicarbonate-Soluble Fraction—The spot of 2-ethoxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone, vanillic acid, and syringic acid as well as two other spots were found on the paper chromatograms.

The Neutral Fraction—This fraction was a yellow viscous syrup containing small amounts of crystals. A single spot giving positive reactions with 2,4-dinitrophenyl-hydrazine (yellowish orange), diazotized sulfanilic acid (yellow), and aqueous ferric chloride (faint dark blue) was found on the paper chromatogram.

Syntheses of Standard Compounds—1-(4-Hydroxy-3-methoxyphenyl)-2-propanone was synthesized by the method of Kulka *et al.* Yellow oil, b.p. 131–133° (0.25–0.30 mm.). Semicarbazone and thio-simicarbazone were prepared and the melting points were 157–158°, and 187–188°, respectively; these melting points were in good agreement with those reported by Kulka *et al.* (*8*).

1-(4-Hydroxy-3,5-dimethoxyphenyl)-2-propanone was synthesized by the similar method. After recrystallization from a mixture of petroleum ether and ether, the compound melted at 68–69°. The semicarbazone melted at (155–156° according to Kulka *et al.*) (*8*).

2-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone was synthesized according to Cramer *et al.* The compound, after recrystallization from benzene, melted at 109–110°. *Analysis*, Calcd. for $C_3H_3O_3(OCH_3)$: OCH_3 15.8, Found: OCH_3 15.6; the melting point of the compound has 109 110° according to Cramer *et al.* (*12*).

Vanilloyl methyl ketone was prepared by the method of Brickman *et al.* (*13*). The compound was recrystallized from dilute ethanol; yellow needles, m.p. 72–73°. The quinoxaline derivative was prepared, m.p. 161–162°, according to Brickman *et al.* the melting points of free compound and its quinoxaline derivative are 72–73° and 161–162°, respectively.

2-Ethoxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone and 1-ethoxy-1-(4-hydroxy-3-methoxyphenyl)-2-propanone were kindly provided by Mr. Ishihara of the Government Forest Experiment Station.

It has been shown that ethanolysis of gymnospermous and angiospermous woods results in a conversion of a part of the lignin constituents into water-soluble oils. Namely, the diketones, vanilloyl methyl ketone and syringoyl methyl ketone, the benzoins, 2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (*14*) and 2-hydroxy-1-(4-hydroxy-3.5-dimethoxyphenyl)-2-propanone (*15*), the desoxybenzoins, 1-(4-hydroxy-3-methoxyphenyl)-2-propanone and 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-propanone, are detected in the bisulfite-soluble fractions from these oils, whereas 2-ethoxy-(4-hydroxy-3-methoxyphenyl)-1-propanone, 2-ethoxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone, and 1-ethoxy-1-(4-hydroxy-3-methoxyphenyl)-2-propanone, 1-ethoxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-2-propanone are identified in the alkaline-soluble fraction. However, such detailed investigations have not hitherto been made on the lignins of monocotyledones. Table III, shows the yields of the ethanolysis products based on the Klason lignin originally present. It can be seen from the Table that yields of the ethanolysis oils obtained

TABLE VI

Compounds Identified in the Water-Soluble Ethanolysis Oils from Bamboo-Stalk

1. Vanillin	R-CHO
2. Syringic	R′-CHO
3. *p*-Hydroxybenzaldehyde	R″-CHO
4. Vanillic acid	R-COOH
5. Syringic acid	R′-COOH
6. 1-(4-Hydroxy-3-methoxyphenyl)-2-propanone	$\mathrm{R\text{-}CH_2 \cdot CO\text{-}CH_3}$
7. 1-(4-Hydroxy-3,5-dimethoxyphenyl)-2-propanone	$\mathrm{R'\text{-}CH_2 \cdot CO\text{-}CH_3}$
8. 2-Hydroxy-1-(4-hydroxy-3-methoxyhenyl)-1-propanone	$\mathrm{R\text{-}CO \cdot CHOH\text{-}CH_3}$
9. 2-Ethoxy-1-(4-hydroxy-3-methoxyphenyl-1-propanone	$\mathrm{R\text{-}CO \cdot CH(OCt)\text{-}CH_3}$
10. 2-Fthoxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone	$\mathrm{R'\text{-}CO \cdot CH(OEt)\text{-}CH_3}$
11. 1-Ethoxy-1-(4-hydroxy-3-methoxyphenyl)-2-propanone	$\mathrm{R\text{-}CH(OEt) \cdot CO\text{-}CH_3}$
12. 1-(4-Hydroxy-3-methoxyphenyl)-1,2.propanedione	$\mathrm{R\text{-}CO \cdot CO\text{-}CH_3}$
13. 1-(4-Hydroxy-3,5-dimethoxyphenyl)-1,2-propanedione	$\mathrm{R'\text{-}CO \cdot CO\text{-}CH_3}$

R: gnaiacyl, R′: syringyl, R″: *p*-hydroxyphenyl.

from bamboo-stalk are comparably higher than those from the gymnosperm. The yields of bisulfite-, bicarbonate- and alkali-soluble fractions and the neutral fraction are shown in Table IV. The yields of these fraction were similar to those which had been obtained from the angiosperms. In Table VI, the compounds identified by paper chromatography and by other analytical methods from the ethanolysis oils of bamboo-stalk are listed. Hibbert *et al.* (*3*) reported that in the ethanolysis oils from bamboo-shoot, 2-ethoxyl-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (in the alkali-soluble fraction) and vanillin (in the bisulfite-soluble fraction) were present, but other compounds could not be detected. They considered therefore that the absence of 2-ethoxy-1-(4-hydroxy-4,5-dimethoxyphenyl)-1-propanone in the alkali-soluble fraction of the ethanolysis oils of bamboo was a single exception. However, in the present study many manomeric phenylpropanes including 2-ethoxy-1-(4-hydroxy-3,5-demethoxyphenyl)-1-propanone have been detected in the ethanolysis oils of bamboo-stalk (Table V). Thus it appears that the lignin of bamboo-stalk is not an exceptional one, and that this lignin is composed of guaiacyl- and syringylpropanes, and presumably also of *p*-hydroxyphenylpropanes.

Hydrolysis of the Material (*16*)—The material (50 g.) suspended in water (500 ml.) was placed into a 500 ml. brass bomb. The bomb was heated for 1 hour at 175°. The reaction mixture was filtered and the filtrate was repeatedly extracted with ether in a separatory funnel. A bisulfite soluble fraction was separated from the ether extract using a 20 per cent sodium bisulfite solution. The identification of the bisulfite soluble fraction was made by comparison of R_f values and color reaction of the sample with those of authentic specimens by paper chromatography. The ascending two dimentional chromatography was run using the aqueous phase of an

TABLE VII

R_f Values and Color Reactions of Compounds Obtained by Hydrolysis of Bamboo-Stalk

Componnd	R_f value		Color reaction	
			Pholoroglucinol-HCl	2,4-Dinitrophenyl-hydrazine-HCl
R′-CH=CH=CHO	0.33[1)]	0.28[2)]	Reddish purple	Yellow orange
R-CH=CH−CHO	0.44	0.40	Reddish purple	Yellow orange
R″-CH=CH−CHO	0.63	0.49	Reddish purple	Yellow orange
R′-CHO	0.31	0.61	Light pink	Dark orange
R-CHO	0.38	0.67	Light pink	Orange
R″-CHO	0.45	0.73	Light pink	Dark orange
Unknown	0.06	0.76	Pale brown	Pale orange

1) *n*-Butanol saturated with 3 per cent NH_4OH.

2) Aqueous layer of a mixture of benzene, methanol and water (50 : 50 : 1 : 50 by volume). R : guaiacyl, R′ : syringyl, R″ : *p*-hydroxyphenyl.

equilibrated mixtures of benzene, ligroin, methanol, and water (50 : 50 : 1 : 50 by volume) and *n*-butanol saturated with 3 per cent NH_4OH respectively. Phloroglucinol-HCl, benzidine-acetic, acid, and 2,4-dinitrophenylhydrazine-HCl were used, respectively, as spraying reagents. The results are shown in Table VII. After spraying with each of these reagents, seven compounds giving distinct colored spots were detected. Of these spots, coniferyl aldehyde, synapyl aldehyde, and *p*-coumaralaldehyde were identified by their R_f values and by specific reddish-purple coloration with phloroglucinol-HCl. Vanillin, syringic aldehyde, and *p*-hydrxybenzaldehyde gave light pink colored spots with phloroglucinol-HCl, whereas with 2,4-dinitrophenylhydrazine-HCl the compounds produced dark orage spots. All seven spots gave dark yellow to light brown colorations with benzidine-acetic acid.

Goldschmid confirmed that vanilloyl methyl ketone and 1-(4-hydroxy-3-methoxyphenyl)-2-propanone were produced in addition to coniferyl aldehyde, *p*-coumaraldehyde, and vanillin upon hydrolysis of hemlock wood and Brauns' hemlock native lignin. The present author has not as yet detected vanilloyl methyl ketone and 1-(4-hydroxy-3-methoxyphenyl)-2-pro panone, but synapyl aldehyde, syringic aldehyde, *p*-hydroxy-benzaldehyde which had not been detected in the hydrolysate of the coniferous lignin have been newly obtained. The formation of these products are in accord with a general idea of the structure of monocotyledonous lignins.

DISCUSSION AND CONCLUSION

Adler (*17*) has recently shown that veratryl glycerol and its β-guaiacyl ether (*18*) are excellent models for certain reactions of lignin such as sulphonation and ethanolysis. Freudenberg and Schlüter (*19*) found very recently that one of the dimers formed as an intermediate in the enzymatic dehydrogenation of coniferyl alcohol is the β-coniferyl ether of guaiacylglycerol, and that the ethanolysis products of the lignins were produced from the β-guaiacyl ether unit in the lignin. The results of ethanolysis and hydrolysis in the present study seem to support the view that a part of the lignin of bamboo-stalk is formed by oxidative condensation through β-guaiacyl ether of guaiacyl-, syringyl, and *p*-hydroxyphenyl propanes; the monomeric products obtained by ethanolysis and hydrolysis may be produced from the β-guaiacyl ether unit. On the other hand, the results of nitrobenzene oxidation would indicate that more than a half of the lignin is presumable combined through C-C linkages which are not cleaved by the oxidation. Further, the fact that Hibbert *et al.* could not find products other than 2-ethoxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone and vanillin from the ethanolysis products of bamboo-shoot may be ascribed either to the use of different species or to the fact that the efficient analytical method of paper chromatography had not been known at that time.

The author wishes to express his sincere thanks to Prof. Dr. I. Kawamura for his kind advice throughout this study.

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The Journal of Biochemistry, Vol, 45, No. 9, 1958

STUDIES ON ARGINASE

I. ENZYMATIC RESOLUTION OF DL-ARGININE INTO ITS OPTICAL ANTIPODES

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(Received for publication, October 2, 1957)

The resolution of racemic arginine into its optical antipodes was first described by Greenstein and his coworkers (*1*). They employed optical specificity of acylase I, obtained from hog kidney, for acetyl-DL-arginine. The present auther has tested crude extract of hog kidney acetone powder and of the commercial Takadiastase preparation for their acylase activities, and found that they are enough active to be satisfactorily utilized as enzyme solution as such for the resolution of acetyl-DL-arginine. Optically pure L- and D-arginines have been obtained in fairly high yields. The present method for resolution has such advantages of Greenstein's that the materials of enzyme are readily available and the preparation of enzyme solution is only to extract the materials.

The difficulties which arose during the course of resolution experiment were in crystallization of acetyl-DL-arginine, the substrate, and in isolation of crystalline acetyl-D-arginine from the incubation mixture with enzyme. It has been found (*2*), however, that crystallization of acetyl-DL-arginine is readily achieved when benzilidine-L-arginine is employed in place of L-arginine as the starting material in acetylation reaction. The procedure is very simple and involves acetylalion, racemization, and splitting of benzilidine linkage in its single step. With crystalline acetyl-DL-arginine thus obtained, the author has found that it is insoluble in cold ethanol and soluble in hot methanol. This characteristic solubility has been satisfactorily utilized for separation of acetyl-D-arginine from L-arginine when these two compounds are present in a mixture incubated with enzyme, thus enabling the isolation of the former compound in crystals* (*2*).

In this communication the descriptions are also made of the use of flavianic acid for the purpose of separating L-arginine as precipitates from admixing acetyl-D-arginine.

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* Recently Birnbaum *et al.* (*3*) reported that acetyl-D-arginine was obtained in high yield and purity merely by concentrating the digestion mixture of acetyl-DL-arginine with acylase I.

EXPERIMENTAL

Substrate

Benzilidien-L-*Arginine*—The material was isolated from acid hydrolyzate of gelatin, edestine, and clupein according to the method of Bergmann and Zervas (*4*). The material contained one molecule of water of crystallization and melted at 207°*.

α-N-Acetyl-DL-*arginine dihydrate*—It was prepared by a simplified technique in which acetylation, racemization**, and removal of benzaldehyde occurred in a single step of the procedure. To a suspension of 15 g. of benzilidine-L-arginine monohydrate in 20 ml. of water was added 30 ml. of acetic anhydride. The suspension was shaken in ice-cold water until the solid dissolved and the mixture was then kept at 37° for 24 hours. After adding about 30 ml. of water to the solution, it was extracted twice with ether to remove the benzaldehyde until the turbid solution was clarified. The acid solution was evaporated *in vacuo* to syrup, which was dissolved in a small amount of water and evaporated again. This procedure was repeated once more to remove acetic acid and solvent. The residue was taken up into about 20 ml. of water, mixed with acetone and kept in an ice-box. On standing overnight acetyl-DL-arginine dihydrate crystallized out in irregularly broken platelets, which were recrystallized from 90 per cent hot methanol. The pure product was optically inactive, melted at 110° and contained two molecules of water of crystallization. The yield was 13 g., or 96 per cent of the theoretical.

Analysis (Kjeldahl). Calcd. for $C_8H_{10}O_3N \cdot 2H_2O$: N, 22.2 per cent
Found: N, 22.1 per cent

Enzymatic Hydrolysis of Acetyl DL-*Arginine by Hog Kidney and Takadiastase Acylase*

Enzyme Preparation—Hog kidney extract was prepared by homogenizing 5 g. of acetone powder of hog kidney with 10 volume of ice-cold water and centrifuging the homogenate at 3000 r.p.m. for 10 minutes. The supernatant fluid was used as acylase preparation.

Takadiastase extract was prepared by macerating 5 g. of the commerical Takadiastase "Sankyo" with 100 ml. of 80 vol. per cent glycerol-water-mixture (pH 8 by addition of NH_4OH) at 37° for 3 hours. The centrifuged supernatant of the extract was dialyzed for 24 hours at 4° against 2 liters of water and the light yellow extract, stored in an ice-box, was employed in the experiments.

Incubation Mixture—A mixture of 4 ml. of *M*/15 phosphate buffer (pH 7 and 8) containing 100 mg. of acetyl-DL-arginine and 1 ml. of enzyme solution was incubated under toluene at 37° for 24 and 48 hours. The blank was run under the same conditions with enzyme solution alone. After incubation, 5 ml. of 10 per cent metaphosphoric acid was added and the mixture was allowed to stand for 20 minutes and filtered. L-Arginine formed in 5 ml. of deproteinized filtrate was determined by the method of Vickery (*6*) which involves the precipitation of arginine with flavianic acid. The hydrolysis values corrected for the blanks are given in Table I and the results show that the breakdown of acetyl-DL-arginine by hog kidney as well as by Takadiastase was limited to 50 per cent of the total value, suggesting asymmetric hydrolysis of the substrate. Fig. 1 indicates that the pH optimum for Takadiastase acylase is nearly at 8.0, which shows a considerably higher value as compared with the pH otimum of 7.2 for hog kidney acylase as reported by Greenstein and his co-workers (*1*). Tests were also made to select the most suitable solvent for the extraction of acylase from Takadiastase preparation. The results obtained are summarized in Table II,

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* All the melting points in this communication are uncorrected.

** Optically active acetyl-L-arginine was yielded from benzilidine-L-arginine when, according to Bergmann and Zervas (*5*), 1.0 mole of the latter was dissolved in 1.5 moles of acetic anhydride and 12 moles of glacial acetic acid and the mixture was heated on a steam bath for 2 hours.

TABLE I

Enzymatic Hydrolysis of Acetyl-DL-Arginine by Hog Kidney and Takadiastase Acylases

Enzyme solution	pH	Hydrolysis value in per cent		
		24 hours	48 hours	72 hours
1 : 10 Aqueous extract of hog kidney acetone powder	7.0	37	48	50
1 : 20 Glycerol extract of Takadiastase	8.0	48	50	50

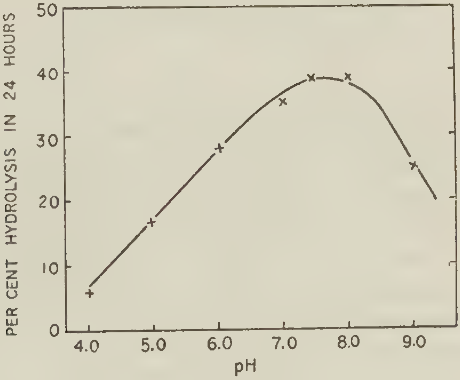

FIG. 1. pH optimum for acylase of aqueous extract of Takadiastase (1 : 20). *M*/15 phosphate buffer (for pH 4–7) and *M*/10 veronal buffer (for pH 8–9) were used.

which demonstrates that the extraction with glycerol yields the highest activity of the Taka-acylase.

TABLE II

Acylase Activity of Takadiastase-Extracts with Various Solvents

(3 per cent extract was used in all cases)

Solvent	Hydrolysis value (%), incubated for 48 hours.
H_2O	20.0
M/15 Phosphate buffer (pH 5.0)	10.0
M/15 Phosphate buffer (pH 6.0)	24.0
M/15 Phosphate buffer (pH 7.0)	24.8
M/10 Veronal buffer (pH 8.0)	33.5
M/10 Veronal buffer (pH 9.0)	18.0
0.9 per cent NaCl	28.0
0.2 per cent NH_4OH	33.1
0.2 per cent CH_3COOH	5.1
70 per cent glycerol (pH 7.0)	42.1

Enzymatic Resolution of DL-*Arginine from Acetyl*-DL-*Argine by Hog Kidney and by Takadiastase*

Incubation Mixture—10 g. of acetyl-DL-arginine dihydrate dissolved in 400 ml. of water was mixed with 100 ml. of enzyme preparation, *i.e.* 20 per cent aqueous homogenate of hog kideny acetone powder or 10 per cent glycerol extract of Takadiastase. The mixture was brought to pH 7.0 in the case of hog kidney or to pH 8.0 in the case of Takadiastase by the addition of dilute NaOH or HCl, and incubated under toluene at 37° for 48 hours. As usual, aliquots were removed from time to follow the course of the digestion and analyzed by the procedure described above. Assays at the about 32nd hour of incubation showed 100 per cent hydrolysis of the susceptible form of the racemic substrate. After complete digestion the mixture was acidfied to pH 4.0 with dilute HCl. and boiled with charcoal for several minutes. The precipitated protein was filtered off by suction and washed several times with cold water. The combined filtrate was evaporated *in va uo* to a syrup, which was again deproteinized by boiling with 300 ml. of 70 per cent methanol.

Separation Method Utilizing Insolubility of Acetyl-D-*Arginine Dihydrate in Cold Ethanol*—The deproteinized methanol solution was brought to pH 2.5 with dilute HCl and evaporated *in vacuo* to a syrup. The residue was dissolved in 50 ml. of hot 95 per cent ethanol and allowed to stand for several days in a refrigerator. The granular crystals of *acetyl*-D-*arginine dihydrate* formed were filtered by suction and washed twice with ice-cold ethanol. The product was recrystallized from hot 95 per cent ethanol. The yield was

4.0 g. or 80 per cent of the theoretical as combined with the second crop. m.p. 115-116°; $[\alpha]_D^{25} = -7.5°$ (*c* 4, H_2O). $[\alpha]_D^{25}$ in literature: +7.0° (*c* 2, *N* HCl) (*3*).

Analysis (Kjeldahl). Calcd. for $C_8H_{10}O_3N \cdot 2H_2O$: N, 22.2 per cent
Found: N, 22.1 per cent

L-*Arginine Monohydrochloride*—The mother liquor and washings of acetyl-D-argine were again warmed with a small amount of charcoal to remove trace of protein, filtered by suction, and the filtrate was adjusted with redistilled aniline to pH 6.0, and evaporated *in vacuo* to a syrup. An ethanol-aceton mixture (1 : 1) (about 100 ml.) was added to the residue and kept in a refrigerator overnight. L-Arginine monohydrochloride which crystallized with concomitant acetyl-D-arginine was filtered by suction and washed repeatedly with 95 per cent ethanol. The dried residue was treated with 50 ml. of hot 95 per cent methanol, and the insoluble L-arginine monohydrochloride was collected by filtration and washed with cold methanol. The yield was 3.5 g. or 83 per cent of the theoretical; m.p. 218–220°; $[\alpha]_D^{25} = +12.1°$ (*c* 2. H_2O), or +22.5° (*c* 2, 5 *N* HCl). $[\alpha]_D$ in literature: +22.80° (*c* 2, 5 *N* HCl) (*1*).

Analysis (Kjeldahl). Calcd. for $C_6H_{15}O_4 \cdot HCl$: N, 26.4 per cent
Found: N, 26.0 per cent

An additional crop of acetyl-D-arginine was obtained by evaporation of the methanolic filtrate *in vacuo* to a syrup, followed by the addition of acetone.

D-*Arginine Monohydrochloride*—One g. of acetyl-D-arginine was refluxed in 20 ml. of 1 *N* HCl for 3 hours and the hydrolyzate was evaporated *in vacuo*. The syrup was dissolved in a small amount of water and the solution was brough to pH 7.0 with aniline, D-Arginine monohydrochloride crystallized out upon adding ethanol to the solution. The yield was 0.76 g. or 92 per cent of the theoretical; m.p. 220°; $[\alpha]_D^{25} = -12.1°$ (*c* 3, H_2O); or −22.0° (*c* 2, 5 *N* HCl). $[\alpha]_D^{25}$ in literature: −23.0° (*c* 2, 5 *N* HCl) (*1*).

Separation of L-*Arginine Flavianate*—To the deproteinized solution mentioned above was added under vigorous stirring 9 g. of flavianic acid in 50 ml. of hot water. L-Arginine flavianate was filtered by suction after standing overnight in an ice-box, and washed with ice-cold water. The yield was about 8.0 g.

L-*Arginine Monohydrochloride*—L-Arginine flavianate was converted to arginine•HCl according to the method of Pratt (*7*). The yield was 3.5 g. or 83 per cent of the theoretical; m.p.0°; 220°$[\alpha]_D^{25} = +12.5°$ (*c* 3, H_2O).

Analysis (Kjeldahl). Calcd. for $C_6H_{15}O_4 \cdot HCl$: N, 26.4 per cent
Found: N, 26.0 per cent

D-*Arginine Flavianate*—The mother liquor and washings from L-arginine removed were combined and evaporated *in vacuo* and the syrupy residue was mixed with 50 ml. of concentrated HCl. After standing overnight in an ice-box, precipitates of excess flavianic acid was filtered off and washed with small amounts of concentrated HCl. The combined filtrate was evaporated *in vacuo* to syrup, which was dissolved in 50 ml. of 2 *N* HCl and refluxed for 2 hours. The hydrolyzate was decolorized with charcoal and evaporated to a syrup. The residue was dissolved in 100 ml. of water and brought to pH 4.0 with dilute NaOH. On adding 9 g. of flavianic acid, 7.5 g. of D-arginine flavianate was obtained.

D-*Arginine Monohydrochloride*—D-Arginine•HCl was isolated in the same way as discribed for L-isomer. The yield was 3.4 g. or 82 per cent of the theoretical; m.p. 218–220°, $[\alpha]_D^{25} = -12.1°$ (c 3, H_2O).

Analysis (Kjeldahl). Calcd. for $C_6H_{15}O_4 \cdot HCl$: N, 26.4 per cent
Found: N, 26.6 per cent

SUMMARY

1. A simple procedure for the preparation of acetyl-DL-arginine from benzilidene-L-arginine in a high yield is described.

2. Acetyl-DL-arginine is asymmetrically hydrolyzed by the extract of hog kidney at pH 7.0 or of Takadiastase (commercial) at pH 8.0 to yield L-arginine and acetyl-D-arginine.

3. Satisfactory separation of the above two products of enzymatic hydrolysis is achieved either by the method utilizing insolubilities of acetyl-D-arginine in acidic ethanol and of L-arginine•HCl in methanol or by the method involving precipitation of arginine by flavianic acid.

The author wishes to express his deep gratitude to Prof. Utzino, M.D., for his interest and encouragement throughout the course of the work.

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The Journal of Biochemistry, Vol. 45, No. 9, 1958

MICROBIOLOGICAL DEGRADATION OF BILE ACIDS

XVI. FORMATION OF 7α-HYDROXY-3,12-DIOXO-Δ^4-CHOLENIC ACID FROM DEHYDROCHOLIC ACID BY CORYNEBACTERIUM SP.

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(Received for publication, December 3, 1957)

Since the publication of the first report (*1*) on the microbiological degradation of cholic acid by means of *Streptomyces gelaticus* 1164, a possible pathway for cholic acid degradation by various microorganisms has been proposed (*2*).

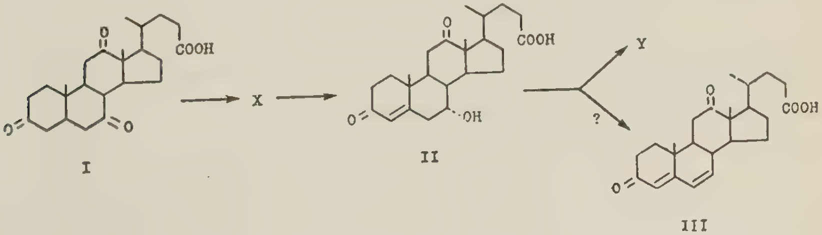
Recently, it has been demonstrated (*3*) that further degradation of dehydrocholic acid, known as an oxidative end product of cholic acid by *Alcaligenes faecalis*, may be performed by *Streptomyces gelaticus* 1164 and a possible pathway for this degradation may be similar to that presumed for the cholic acid degradation by the same microbe.

In a previous paper of this series, Eguchi (*4*) has reported that a bacterium, isolated from the soil of a slaughter-yard, is able to grow on the medium containing cholic acid as the sole source of carbon and convert the cholic acid into 7α, 12α-dihydroxy-3-oxo-Δ^4-cholenic acid. This microorganism has been subsequently identified as *Corynebacterium* sp. and it is quite likely to be *Corynebacterium equi* (*5*).

It then became of interest to expand our studies to the comparison between cholic acid and dehydrocholic acid degradations by the above *Corynebacterium* sp. In the present study, therefore, separation and identification of the degradation products of dehydrocholic acid by *Corynebacterium* sp. were carried out and four intermediate products were isolated as crystals.

Diagram 1

Proposed degradation pathway of dehydrocholic acid by *Corynebacterium* sp.



Only two out of four were identified to be 7α-hydroxy-3,12-dioxo-Δ^4-cholenic acid and 3,12-dioxo-$\Delta^{4,6}$-choladienic acid.

It is of interest that both *Streptomyces gelaticus* 1164 (*3*) and *Corynebacterium* sp. are able to form 7α-hydroxy-3,12-dioxo-Δ^4-cholenic acid (II) from dehydrocholic acid (I).

On the basis of this finding, an oxidative pathway of dehydrocholic acid by *Corynebacterium* sp. may be deduced as shown in Diagram 1, though the intermediates X and Y are not yet isolated.

EXPERIMENTAL

Cultivation—2 g. of dehydrocholic acid was dissolved in a mineral solution (K_2CO_3 0.6 g.; $(NH_4)_2SO_4$ 2 g.; K_2HPO_4 1 g.; $MgSO_4 \cdot 7H_2O$ 0.5 g.; $FeCl_3 \cdot 6H_2O$ 0.01 g.; tap water 1000 ml.) and the pH of the resulting solution was adjusted to 7.2. This synthetic medium was autoclaved at 110° for 20 minutes. After inoculation of *Corynebacterium* sp., the medium was incubated at 37° for 8 or 10 days.

After the incubation for 2 or 4 days, an appearence of violet fluorescence in the culture was observed as seen in the case of the dehydrocholic acid degradation by *Streptomyces gelaticus* 1164. Its fluorescence is probably due to the formation of a compound related to Shimizu-Mizuhara's fluorescence test (*6*) for dehydrocholic acid.

Separation of 7α-Hydroxy-3,12-Dioxo-Δ⁴-Cholenic Acid—6 g. of dehydrocholic acid was used for this experiment, and incubation mixture was warmed at about 70° and then blown to about one-tenth volume of the original in a stream of air. To the concentrated culture medium was added 2 volumes of ethanol and the mixture was left standing overnight in the cold and filtered. The filtrate was evaporated to dryness in vacuum below 40°. The residue was dissolved in about 50 ml. of 0.5 per cent sodium bicarbonate solution, acidified (about pH 3.0) with dilute hydrochloric acid and left standing overnight in the cold. The resultant precipitate was filtered, washed with water, dried and gave 2.7 g. of pale brown powder.

The powder was covered with a thin layer of ethyl acetate. After crystallization appeared, the crystals were filtered, recrystallized from methanol and afforded 0.9 g. of plates melting at 246–249° with decomposition, $\lambda_{\max}^{\text{alc.}}$ 241 mμ. (log ε, 4.21). The infrared spectrum showed the following absorption bands (in Nujol): hydroxyl, 3.02 μ; carboxyl, 5.82 μ; C_{12}-ketone, 5.88 μ; Δ^4-3-ketone, 6.04 and 6.18 μ. The melting point of 7α-hydroxy-3,12-dioxo-Δ^4-cholenic acid, isolated in a previous paper (*3*), was 224–226° (decomp.) and different from one of the acid isolated here, but those infrared absorption spectra were identical.

Analysis Calcd. for $C_{24}H_{34}O_5$: C, 71.61; H, 8.51.
Found: C, 72.16; H, 8.28.

The methyl ester of this acid was prepared with diazomethane by the usual manner and recrystallized from ethyl acetate-petroleum ether, m.p.191–193° without decomposition.

Analysis Calcd. for $C_{25}H_{36}O_5$: C, 72.16; H, 8.56.
Found: C, 72.17; H, 8.56.

To confirm the constitution of this ester, it was converted to an authentic methyl 3,12-dioxo-$\Delta^{4,6}$-choladienate (*7*) by the following manner.

Methyl 3,12-Dioxo-$\Delta^{4,6}$-Choladienate—A solution of 60 mg. of the above ester, m.p. 191–193°, in 6 ml. of methanol contaning three drops of concentrated sulfuric acid was refluxed on a boiling water bath for 20 minutes. The resultant pale yellow solution was neutralized and diluted with dilute sodium bicarbonate solution. The crystalline precipitate which was separated on standing, was collected, washed with water and dried. Crystallization of the dried precipitate from ethyl acetate-petroleum ether afforded 47 mg. of pale yellow prismatic needles of m.p. 175–177°, $\lambda_{\max}^{\text{alc.}}$ 280.5 mμ (log ε, 4.42), and $\lambda_{\max}^{\text{Nujol}}$ 5.81 μ (ester), 5.85 μ (C_{12}-ketone) and 6.00, 6.18 and 6.30 μ ($\Delta^{4,6}$-3-ketone). Identity with an authentic sample (*7*) with m.p. 175–177° was demonstrated by mixed melting point determination and infrared comparison.

Analysis Calcd. for $C_{25}H_{34}O_4$: C, 75.34; H, 8.60.
Found: C, 75.15; H, 8.55.

Chromatographic Separation of the Various Intermediates—The original ethyl acetate and methanol filtrates, obtained after the above-described separation of 7α-hydroxy-2,12-dioxo-Δ^4-cholenic acid, were evaporated to dryness and gave 1.6 g. of a pale brown gum containing a small amount of crystals. This gum was dissolved in methanol and esterified with diazomethane in ether by the usual manner. After evaporation of solvents, the residue was dissolved in 20 ml. of ether. Soon the crystals separated out from the ether solution. This compound was filtered, washed with ether and recrystallized from ethyl acetate-petroleum ether and gave 12 mg. of methyl 7α-hydroxy-3,12-dioxo-Δ^4-cholenate.

The ether filtrate and washings were combined and concentrated to about 20 ml. The concentrated ether solution was poured onto 10 g. of active alumina (Wako, 200 mesh) column of dimensions 130×11 mm. Various fractions were eluted with several solvents as shown in Table I, and the four kinds of methyl esters were separated.

Fraction No. 3 and 4 gave prismatic needles with m.p. 172–174°. The characterization of this ester as methyl 3,12-dioxo-$\Delta^{4,6}$-choladienate was confirmed by mixed melting point determination and comparison of the infrared spectrum.

Analysis Calcd. for $C_{25}H_{34}O_4$: C, 75.34; H, 8.60.
Found: C, 75.48; H, 8.78.

Fraction No. 5 and 6 gave needles sintering at 190° and melting at 202–204°. The identification of this ester was not performed, but the infrared spectrum of this ester showed no hydroxyl absorption, but ester (5.76 and 8.55 μ) and six-membered cyclic ketone (5.89 μ).

Spectrophotometric Analysis of Degradation Products—The above-descrived synthetic medium which was adopted for the isolation of the degradation products of dehydrocholic acid was used. After inoculation of *Corynebacterium* sp., an aliquot of the culture medium was diluted (1 :100) and centrifuged at 4,000 r.p.m. for 15 minutes. The ultraviolet absorption spectra of the supernatants were measured by Beckman spectrophotometer, type DK I. The data are shown in Fig. 1.

Table I

Chromatographic Details for the Separation of Dehydrocholic Acid Metabolites Obtained after Incubation of Dehydrocholic Acid with Corynebacterium Sp.

Fraction number	Solvents of elution	Yields of Crystals* (*mg.*)	m.p. (C)	Identified substances**	$\lambda_{max}^{alc.}$ of crystals (mμ)
1	Ether (10 *ml.*)	None	—	—	—
2	,, (,,)	Trace***	170–182°	Unidentified	None****
3 4	,, (,,) ,, (,,)	40	172–174°	Methyl 3,12-dioxo-$\Delta^{4,6}$-choladienate	282
5 6	,, (20 *ml.*) Ethyl acetate (10 *ml.*)	10	202–204°	Unidentified	None****
7	,, (,,)	oil	—	Methyl 7α-hydroxy-3,12-dioxo-Δ^4-cholenate	—
8	Acetone (,,)	14	188–190°	—	241
9	,, (,,)	4***	240–245° (decomp.)	Unidentified	240–241
10	Methanol (20 *ml.*)	oil	—	—	—
11	,, (,,)	oil	—	—	—

* Each elute was evaporated to dryness and the each resulting residue was crystallized from ethyl acetate-petroleum ether. Oil means giving no crystals after such treatments.

** See text for experimental details.

*** These substances require further purification, so those m.ps. mean no exact values.

**** This showed no characteristic absorption in the region of 220 to 330 mμ.

DISCUSSION

7α-Hydroxy-3,12-dioxo-Δ^4-cholenic acid (II) and 3,12-dioxo-$\Delta^{4,6}$-choladienic acid (III) were obtained as the degradation product of dehydrocholic acid (I) by *Corynebacterium* sp. It is well known that a 7α-hydroxy-3-ketone structure is easily converted to a $\Delta^{4,6}$-3-ketone by mineral acid or caustic alkali. So it is of interest to decide whether the acid III is formed from the acid II by an enzymatic reaction or by an artificial transformation inevitaly accompanied in the isolation process.

In order to solve such question, ultraviolet absorption spectra of the incubation mixture are measured. As seen in Fig. 1, there is only one peak at 246 mμ which is the specific absorption maximum for the acid II. But no absorption is observed at around 290 mμ which is specific for the acid III.

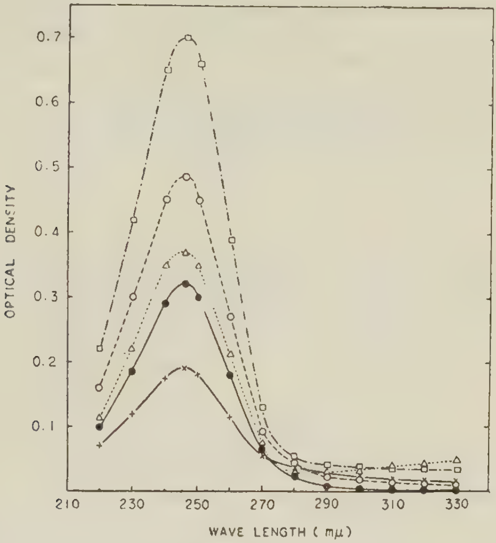


FIG. 1. Ultraviolet absorption spectra of the culture supernatant (diluted 1 : 100) obtained after incubation of dehydrocholic acid (0.2 g./dl.) with *Corynebacterium* sp. at 37° for the following incubation periods: —×—, 3 days; ···△···, 4 days; –·–□–·–, 6 days; – –○– –, 9 days; —●—, 13 days.

Thus, the acid III may be not transformed microbiologically from the acid II but may be an artificial product accompanied by the isolation process.

SUMMARY

Dehydrocholic acid was incubated with *Corynebacterium* sp. in the synthetic medium containing this bile acid as the sole source of carbon and four transformation products were isolated as crystals. Two out of them were identified 7α-hydroxy-3,12-dioxo-Δ^{4}-cholenic acid and 3,12-dioxo-$\Delta^{4,6}$-choladienic acid. However, ultraviolet absorption data of the culture suggests that the latter dienone acid may not be a microbiological transformation product.

The author wishes to express his sincere thanks to Prof. T. Shimizu, Prof. S. Mizuhara and Dr. S. Hayakawa for their kind guidance throughout this research, and to Mr. Y. Matsui of Research Laboratory, Shionogi & Co., Ltd. for his help in measuring the infrared absorption spectra and for his valuable criticism.

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The Journal of Biochemistry, Vol. 45, No. 9, 1958

INACTIVATION OF SERUM TRYPSIN-INHIBITOR CAUSED BY BILE AND BILE ACIDS

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(Received for publication, November 22, 1957)

The naturally occurring enzyme inhibitors have frequently been reported to exist in various animal and plant tissues. Change in the activity of these inhibitors in a biological system may be worth consideration. In the course of studies on the action of trypsin-inhibitor in serum, author has incidentally found a definite action of bile on the inhibitor. The present paper deals with this phase of investigation.

Although the exact nature of trypsin-inhibitor in serum is still unknown, it is supposed to be proteinacious high molecular weight substance. Detailed studies on the interaction between trypsin and serum inhibitor were carried out by McCann and Laskowsky (*1*), who reported that the inhibitor formed stoichiometrically a non-dissociable complex with trypsin. Author's first attempt was made to fined out substances which affect the trypsin-inhibitor. Some of the substances tested, such as chloroform, ethyl alcohol, lauryltrimethylammonium chloride and bovine bile, were found to be effective in inactivating the inhibitor. Of these active substances bovine bile seemed especially to be interesting because of its biological nature. The following report describes the details of the interaction of the bile with serum trypsin-inhibitor.

MATERIALS AND METHODS

Trypsin was prepared after the method of Kunitz and Northrop (*2*). Untreated fresh horse serum was found to be satisfactory as a trypsin-inhibitor preparation. Commercially available bovine bile powder, bile acids and pigment were used as test substances. 3,12-Diketocholanic acid and 3,7-diketocholanic acid were kindly supplied by Dr. S. Hayakawa of Okayama University. Casein after Hammarsten was used as the substrate of enzyme.

Experimental system used in this experiment was as follows unless otherwise stated: after the incubation of 0.5 ml. of 2 per cent (*v/v*) horse serum with 0.5 ml. of bile or bile acid solution, 0.1 ml. of enzyme solution was added and allowed to stand for 5 minutes to bring the reaction between inhibitor and trypsin in completion. Three ml. of 1 per cent casein, a substrate, was then added to the mixture. Each of these solution was prepared using $M/20$ borate buffer of pH 8.0. The reaction mixture was kept at 25° and at intervals aliquots were taken out from the mixture and the tryptic activity of each aliquot was estimated by the method of Herriott (*2*).

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RESULTS

Table I shows the effect of a definite concentration (2 per cent serum) of trypsin-inhibitor on the activity of trypsin in the presence of different concentrations of bovine bile. The degree of suppression of the inhibitory activity (I) was calculated by an equation,

$$I=\frac{V_b-V_i}{V_0-V_i}\times 100\%$$

where V_0 and V_i represent the rate of tryptic activity in the absence and the presence of serum, respectively, and V_b in the presence of serum treated with bile or related substances.

Table I

Effect of Bile on Serum Trypsin-Inhitor

Alll experiments were carried out at pH 8.0 and 25°. Tryptic activity was expressed in terms of micromoles of tyrosine formed per ml. of reaction mixture after 30 minutes hydrolysis.

	Conc. of bile %	Tyrosine liberated $\mu M/ml.$	Decrease in inhibitory activity %
Trypsin alone	—	0.31	—
Trypsin+bile	5.0	0.29	—
Trypsin+serum treated with bile	5.0	0.15	46
,, ,,	2.5	0.12	34
,, ,,	0.5	0.08	18
Trypsin+untreated serum	—	0.03	0

As seen from Table I the bile showed a definite effect on the activity of serum inhibitor. Therefore, the effect of the individual components of bile and some related compounds on the reaction between trypsin and serum inhibitor were tested. It should be mentioned that bile acids showed an appreciable effect on the tryptic digestion. Fifteen per cent reduction in trypsin activity was caused by $1.5\times10^{-2}\,M$ sodium desoxycholate. Therefore, a control experiment (trypsin+test substance) was ran in each experiment to check the effect of test substance on trypsin activity. Of the bile acids tested, marked reduction in the inhibitory powder of serum inhibitor could thus be found only in the case of desoxycholic acid. Cholic acid, taurocholic acid and glycocholic acid were shown to be slightly effective at their higher concentrations. Results are summarized in Table II.

The reaction of serum inhibitor and trypsin was independent of the pH of the solution within a wide range between pH 6 and 9. On the other hand, the rate of inactivation of inhibitor by desoxycholate was found to be highly dependent on pH of the reaction system. These results are given in Table III. Since the solubility of desoxycholic acid dropped below pH 6, experiments were mostly carried out at alkaline pH's. The most marked

TABLE II

Effect of the Components of Bile and Related Compounds on Serum Trypsin-inhibitor

Compound⁽¹⁾	Concentration M	Decrease in inhibitory activity (%)
Cholic acid	10^{-2}	0
,,	5×10^{-2}	17
Desoxycholic acid	10^{-2}	70
Dehydrocholic acid	2×10^{-2}	0
3,12-Diketocholanic acid	2×10^{-2}	0
3,7-Diketocholanic acid	2×10^{-2}	0
Glycholic acid	2×10^{-2}	18
Glycine	2×10^{-2}	0
Taurocholic acid	2×10^{-2}	16
Taurine	2×10^{-2}	0
Aceto-choleic acid⁽²⁾ (1 acetic acid and 1 desoxycholic acid)	10^{-2}	68
Propio-choleic acid⁽²⁾ (1 propionic acid and 3 desoxycholic acid)	2.5×10^{-3}	60
Palmitic-choleic acid⁽²⁾ (1 palmitic acid and 8 desoxycholic acid)	2.5×10^{-3}	60
Bilirubin⁽³⁾	10^{-4}	—

1) All compounds were used as sodium salt.

2) The constitutions of choleic acids are shown in parentheses.

3) Inhibition of trypsin by bilirubin is so strong that that the effect on inhibitor could not be determined.

TABLE III

Effect of pH on the Action of Sodium Desoxycholate

Concentration of desoxycholate: 10^{-2} M

pH	Percentage decrease in inhibitory activity
9.9	24
9.0	42
8.1	67
7.1	90
6.5	95

inactivation was found to occur at pH 6.5 to 7.0.

The reversibility of the inactivation of trypsin-inhibitor caused by desoxycholate was studied. Horse serum was incubated together with sodium desoxycholate at pH 8.1 at 25° for 20 minutes and the mixture was divided into four equal parts. The activity of trypsin-inhibitor the first part was estimated immediately. The second and third parts were dialyzed for 24 hours at 5° against borate buffer at pH 8.1 and 9.9, respectively. The last part was allowed to stand for 24 hours at 5°. As shown in Table IV, any recovery of inhibitory activity could not be observed. Further evidence for the irreversibility of the interaction is shown in Table V. In this experiment the effect of dilution of the mixture of inhibitor and desoxycholate was examined. One ml. of 20 per cent (v/v) horse serum treated with sodium desoxycholate ($2\times10^{-2}\,M$) for 20 minutes was diluted to the volume indicated in the table. One ml. of each diluent was taken out and the inhibitory activity was determined as mentioned previously. In this experiment, at the final concentration of desoxycholate it showed an effect only slightly, if any. Thus it appeared that the inactivated inhibitor could not be restored by dilution.

TABLE IV

Effect of Dialysis on Serum Inhibitor Treated with Desoxycholate

Concentration of desoxycholate: $8\times10^{-3}\,M$

No.		Percentage decrease in inhibitory activity
1	Before dialysis	55
2	Dialyzed against borate buffer at pH 8.1	63
3	Dialyzed against borate buffer at pH 9.9	60
4	Not dialyzed, allowed to stand under the same condition	58

TABLE V

Effect of Dilution of the Mixture of Inhibitor and Sodium Desoxycholate

One ml. of each diluent prepared as described in the text was assayed after the addition of 0.1 ml. enzyme and 3 ml. substrate solution.

Volume of diluent (*ml.*)	Percentage decrease in inhibitory activity
6.0	55
8.0	60
10.0	59
12.0	57

Another experiment was designed to follow the time course of the interaction between serum inhibitor and desoxycholate. Serum was allowed to react with desoxycholate at pH 8.0 and 25°. At various time intervals aliquots were taken and the loss of inhibitory activity was estimated. As shown in Fig. 1, inactivation reaction proceeded very rapidly during the initial short period, followed by a constant slow rate reaction lasting for several hours. Thus, it seemed that the loss of the inhibitory activity in the presence of desoxycholate continued for a long period until it reached 100 per cent decrease.

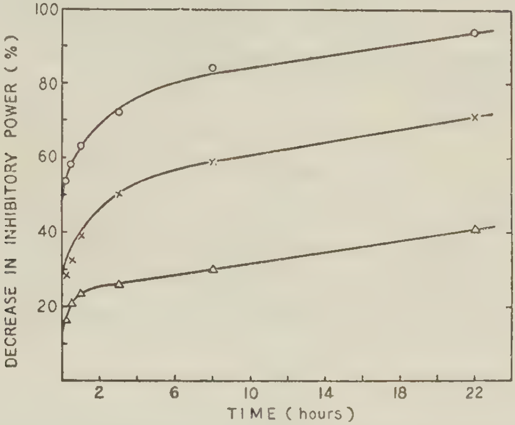

Fig. 1. Time course of the interaction between serum inhibitor and desoxycholate. Concentrations of desoxycholate used were as follow: $7.5 \times 10^{-3} M$ (—○—), $5.00 \times 10^{-3} M$ (—×—), $3.7 \times 10^{-3} M$ (—△—).

Table VI

Effect of Sequence of Mixing Reactants

Conc. of desoxycholate ($10^{-3} M$)	Percentage decrease in inhibitory activity*		
	(I+DCA)+T	(T+DCA)+I	(T+I)+DCA
2.2	11	2	0
5.0	38	7	0
9.0	69	10	0
15.0	84	18	0

* T, I and DCA represent trypsin, inhibitor and desoxycholate, respectively. Two substances in parentheses were first mixed together followed by the addition of the third substance.

On testing the interaction among trypsin, inhibitor and desoxycholate, the sequence of addition of these components were found to be critical (Table VI). When desoxycholate was added to the mixture of trypsin and inhibitor, *i.e.*, inhibitor-trypsin compound, inactivation could not be observed at any rate, while the addition of trypsin to the mixture of inhibitor and desoxycholate showed a conspicuous inactivation. In the case in which the inhibitor was added to the mixture of trypsin and desoxycholate, only a slight inactivation was obtained. These observations suggest that the rate of the reaction between trypsin and inhibitor is far higher than that between the inhibitor and desoxycholate.

In order to examine whether or not the action of desoxycholate is specific to serum trypsin-inhibitor, soy bean inhibitor prepared after the method of Kunitz (*2*) was employed instead of serum inhibitor. However, no inactivation of the inhibitory power could be demonstrated. This fact seems to indicate that the action of desoxycholate may be limited at least to the serum inhibitor.

DISCUSSION

It was disclosed that the inactivation of serum trypsin-inhibitor caused by bovine bile was mostly due to desoxycholic acid. Two general alternative mechanisms in the interaction may be considered. The one possibility would be to assume that the reaction proceeds to the formation of inactive complex as follows:

$$\mathrm{T}+\mathrm{I}\longrightarrow\mathrm{TI} \qquad (1) \text{ and}$$

$$\mathrm{I}+n\mathrm{B}\longrightarrow\mathrm{IB}_n \qquad (2)$$

where T, I and B are trypsin, inhibitor and desoxycholate, respectively. n is the number of molecules of desoxycholate required to convert 1 unit of inhibitor into the inactive form. In the reaction (*2*), it is necessary to assume that the inactive complex IB_n involves the active center of inhibitor. The second possibility is that the denaturation of inhibitor molecule caused by desoxycholate is responsible for the inactivation. According to Anson (*3*), indeed, bile salts have a denaturing action on a certain protein. The analysis of the reaction between the inhibitor and desoxycholate (Fig. 1) suggests that the reaction involves a reversible chemical combination, reaching completion in a few minutes, followed (or accompanied) by a slower irreversible denaturation. In the initial phase of reaction, an attempt was made to evaluate approximately the equilibrium constant (K_{B}) and value n in reaction (*2*). From this relationship the following equation can be derived:

$$\log\frac{a}{1+a}+n\log[\mathrm{B}]=\log K_{\mathrm{B}}$$

in which a is the concentration of active inhibitor. It can be calculated from the results shown in Fig. 2 that K_{B} is $10^{-4.4}$ mole/liter and n is 2 at 25°, pH 8.0. Based on the fact that the affinity between inhibitor and trypsin is very much higher than that between inhibitor and desoxycholate, it may be reasonable to assume that trypsin occupies the active center of inhibitor faster than desoxycholate.

Worth mentioning is the finding by Wattenberg and Glick (*4*) that the activity of hyaluronidase inhibitor in serum is largely reduced in the presence of bile salts or hemoglobin derivatives. In consideration of the *in vivo* activity of bile salts in inactivating enzyme-inhibitor in serum, the concentration of these substances in serum may be too low to be significant. Nevertheless, the combined effect of these compounds on enzyme-inhibitor system might be of physiological significance, particulary when the levels of these substances might increase.

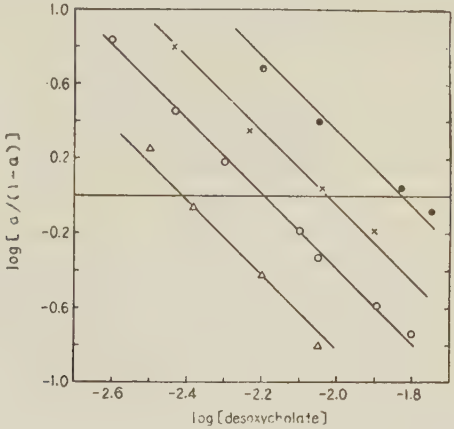

Fig. 2. Effect of concentration of desoxycholate on inhibitory activity of serum at various temperatures. —●— 13°, —×— 20°, —○— 25,° —△— 30°

SUMMARY

1. Inactivation of serum trypsin-inhibitor caused by bile and bile acids was investigated. Desoxycholic acid was most effective among various bile acids tested.

2. The inactivation reaction caused by desoxycholate could not be reversed either by dilution or by change in pH.

3. Desoxycholate could not recover the trypsin activity from inhibitor-trypsin complex. Inactivation reaction is, presumably, due to the combination of two molecules of desoxycholate with inhibitor.

Author wishes to thank Prof. H. Tamiya and Prof. T. Yanagita for their valuable suggestions and encouragement in this work.

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The Journal of Biochemistry, Vol. 45, No. 9, 1958

TERMINAL OXIDATION SYSTEM IN RESPIRING YEAST

II. RE-EVALUATION OF ROLE OF ACTION OF CYTOCHROME B_2 IN DEHYDROGENATIONS OF LACTATE AND MALATE

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In 1954, Appleby and Morton (*1*) reported on the crystallization of lactic dehydrogenase from baker's yeast, which contained cytochrome b_2 and flavin mononucleotide (FMN) and could catalyze the reduction of cytochrome c by lactate without addition of coenzyme. On the other hand, we have succeeded in preparing crystalline cytochrome b_2 from baker's yeast, which does not show any enzymatic activity and contains no flavin. In the previous study on the role of action of cytochrome b_2 in respiring yeast (*2*), we have proposed a scheme of electron-transferring system such as given below;

$$\begin{tikzcd} \text{Lactate} \arrow[drr, "\text{Dehydrogenase}"] & & & \\ \text{Malate} \arrow[rr, "\text{Dehydrogenase}"] & & \text{Cytochrome } b_2 \arrow[r] & \text{Cytochrome c} \\ \text{TPNH} \arrow[urr, "\text{Dehydrogenase}"] & & & \end{tikzcd}$$

Our preparation of yeast lactic dehydrogenase, which had been fairly purified according to the method of Bach, Dixon, and Zerfas (*3*) with some modification, contained at least cytochrome b_2 and a flavin, and when lactate, malate or reduced triphosphopyridine nucleotide (TPNH) was added to the enzyme preparation, the cytochrome b_2 contained in the preparation, could be immediately reduced, though there were notable differences in the reducibility of each substrate.

In this paper, the role of action of cytochrome b_2 contained in the enzyme preparation is re-evaluated by the study in which cytochrome b_2 is used as the final electron acceptor of yeast lactic dehyrogenase, and the mode of existence of cytochrome b_2 will be discussed with regard to a protein complex of lactic dehydrogenase moiety and cytochrome b_2 moiety.

MATERIALS AND METHODS

Preparation of Crystalline Cytochrome c from Baker's Yeast—This was carried out according to the method reported in our previous papers (*4*, *5*).

Preparation of Crystalline Cytochrome b_2 from Baker's Yeast—This was carried out according to our method previously described (*6*).

Preparation of Yeast Lactic Dehydrogenase—This was carried out according to the method described in our previous paper (*2*). This enzyme preparation contains cytochrome b_2 and a certain flavin together with the lactic dehydrogenase activity but not cytochrome c. During the preparation procedure, almost all of cytochrome b_2 was adsorbed to calcium phosphate gel from the dialyzed crude yeast extract, irrespective as to whether it was bound to yeast lactic dehydrogenase or not. Cytochrome b_2 (not bound to the dehydrogenase) was easily liberated by washing the gel with $M/15$ phosphate buffer of pH 7.2, while the cytochrome b_2 bound to the dehydrogenase was liberated with the buffer containing 20 per cent saturation of ammonium sulfate.

Chemical Reagent—Flavin adenine dinucleotide (90 per cent in purity, FAD) was a kind gift from Mr. M. Yoneda in Takeda Pharmaceutical Co., Ltd., Osaka.

Spectrophotometric Analysis—This was carried out with the use of Shimadzu photoelectric spectrophotometer, type QB-50, using a silica cuvette of 1 cm. light path. Anaerobic measurement was performed in nitrogen gas with the use of Thunberg's tube-type of a glass cuvette of 1 cml. light path having a side arm.

RESULTS

Reductions of Cytochrome b_2 by Lactate and by Malate in the Presence of the Preparation of Yeast Lactic Dehydrogenase—Appleby and Morton (*1*) reported that the crystalline yeast lactic dehydrogenase could reduce cytochrome c prepared from a certain source. On the other hand, we (*6*) have succeeded in preparing crystalline cytochrome b_2 from baker's yeast; the crystals have no lactic dehydrogenase activity and no flavin, and show absorption maxima at 278, 359, 411 and 563 mμ in the oxidized state, and at 422, 528 and 557 mμ in the dithionite-reduced state. This absorption spectrum except that around 278 mμ is extremely similar to that of the yeast lactic dehydrogenase crystallized by Appleby and Morton (*1*) and fairly purified by us, indicating that cytochrome b_2 is able to exist as one protein unit as well as the cytochrome c's crystallized by us from baker's yeast, bovine heart muscle and pig heart muscle (*4, 5, 7*). These observations suggest that the yeast lactic dehydrogenase consists of some moieties; at least, lactic dehydrogenase moiety, flavin moiety and cytochrome b_2 moiety, forming a protein complex. We (*2*) have recently reported that the preparation of yeast lactic dehydrogenase fairly purified can catalyze the reductions of yeast cytochrome c by lactate, by malate and by TPNH, and the cytochrome b_2 contained in the enzyme preparation is immediately reduced by each addition of these three substrates. Therefore, in order to further confirm the reduction of the cytochrome b_2 moiety of the enzyme by these substrates, cytochrome b_2 was used as the final electron acceptor instead of cytochrome c. Since thus far only cytochrome b_2 moiety could be separated from the protein complex of yeast lactic dehydrogenase, the whole of the yeast lactic dehydrogenase preparation was used as the electron-donating enzyme to cytochrome b_2. Cytochrome b_2 can be reduced by lactate and by malate in the presence of the enzyme preparation, as shown in Fig. 1. The reduction of cytochrome b_2 by malate is notable slow compared with that by lactate, at pH 6.4, as well as in the case when cytochrome c is used at the final electron acceptor.

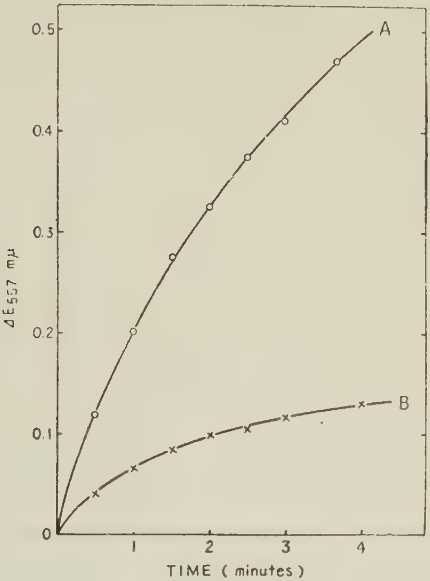


FIG. 1. Reductions of cytochrome b_2 by lactate and by malate in the presence of yeast lactic dehydrogenase preparation.

The components of reaction mixture were as follow; 0.1 *M* phosphate buffer of pH 6.4. 0.1 *M* sodium lactare or sodium malate (pH 6.4), cytochrome b_2, the yeast lactic dehydrogenase preparation in 4.0 ml. of the total volume. The reaction was started by adding the enzyme solution and carried out in nitrogen gas at a room temperature. Curve A, lactate; B, malate.

Autoxidizability of Cytochrome b_2—When the reduction of cytochrome b_2 is carried out in the air in the presence of lactate and the preparation of yeast lactic dehydrogenase, the reduction of cytochrome b_2 is not observable. As shown in Fig. 2, the cytochrome b_2 reduced enzymically in vacuum is swiftly oxidized by the air introduced into the Thunberg's tube-type of cuvette. This fact indicates that cytochrome b_2 has a remarkable autoxidizability.

Influence of pH on Reductions of Cytochrome b_2 by Lactate and by Malate in the Presence of the Preparation of Yeast Lactic Dehydrogenase—As already reported (*2*), the optimum pH in the reductions of yeast cytochrome c by lactate and by malate in the presence of the enzyme preparation was 6.5 and 8.0, respectively. However, when cytochrome b_2 is used as the final electron acceptor instead of yeast cytochrome c, notably different results are obtained, as shown in Fig. 3. In the case of lactate, the activity does not decrease at pH 4.0 so remarkably as in the case of the reduction of cytochrome c by lactate, and in the case of malate, the activity does not decrease even at pH 9.0 at all.

Effects of Flavins on Reduction of Cytochrome b_2 *by Lactate in the Presence of the Preparation of Yeast Lactic Dehydrogenase*—Boeri, Cutolo, Luzzati

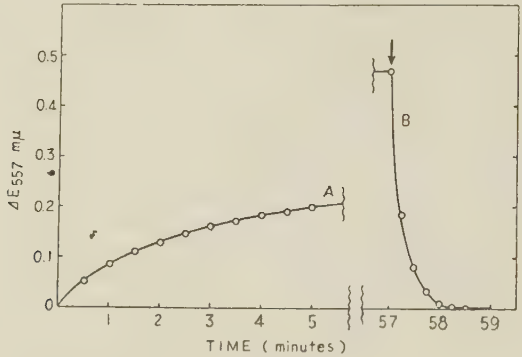


FIG. 2. Autoxidizability of cytochrome b_2.

The experimental condition was same as for Fig. 1, except that phosphate buffer of pH 6.0 was used, and the reaction during the first 57 minutes was carried out in a reduced pressure (5 mm. Hg.) and at the time shown by an arrow, air was introduced into the Thunberg's tube-type of cuvette. Curve A, reduction of cytochrome b_2 in a reduced pressure; B, oxidation of cytochrome b_2 in the air.

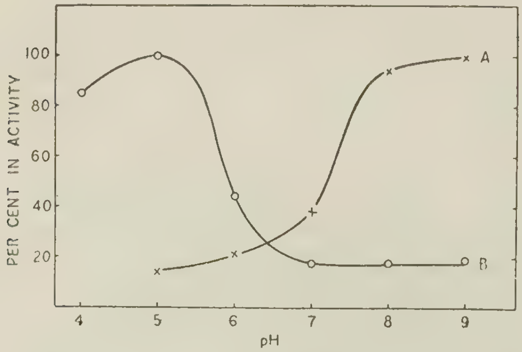


FIG. 3. Influence of pH on reductions of cytochrome b_2 by lactate and by malate in the presence of yeast lactic dehydrogenase preparation.

The experimental condition was same as for Fig. 1, except that phosphate buffers of various pH were used. "Percent in activity" was calculated as each maximum activity in both substrates was regarded as 100. Curve A, for malate; B, for lactate.

and Tosi (*8*), and Appleby and Morton (*1*) found that in their best preparation of yeast lactic dehydrogenase, there existed a flavin which was identified as FMN. Then Boeri and Tosi (*9*) proposed a scheme with regard to the electron transport from lactate to the cytochrome b_2 moiety of the yeast lactic dehydrogenase, where the electrons liberated from lactate are transfered at first to the FMN-moiety and finally to the cytochrome b_2 moiety. In fact, cytochrome b_2 can be spontaneously reduced by reduced FMN. As already described (*6*), the reduction of cytochrome b_2 by lactate in the presence of the yeast lactic dehydrogenase preparation is slightly accelerated in the presence of FMN as the appearance of the α-absorption band of cytochrome b_2 is determined by a microspectroscopic observation. However, when the reduction of cytochrome b_2 is spectrophotometrically

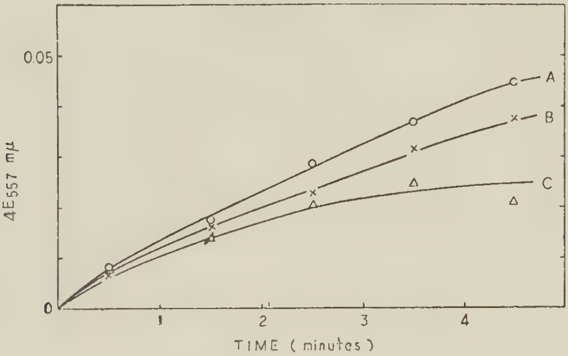


FIG. 4. Effect of FMN on reduction of cytochrome b_2 by lactate in the presence of yeast lactic dehydrogenase preparation.

The experimental condition was same as for Fig. 1, except that FMN was added to the reaction mixture. Curve A, lactate; B, $+10^{-6}$ *M* FMN; C, $+10^{-4}$ *M* FMN.

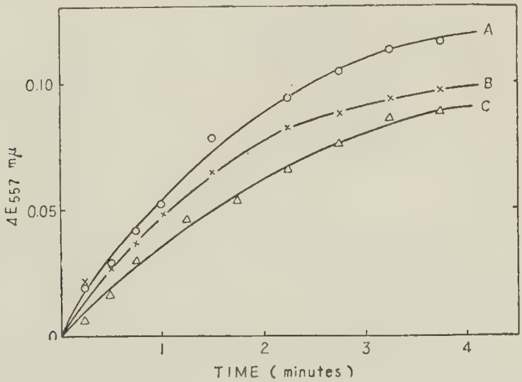


FIG. 5. Effect of FAD on reduction of cytochrome b_2 by lactate in the presence of yeast lactic dehydrogenase preparation.

The experimental condition was same as for Fig. 1, except that FAD was added to the reaction mixture. Curve A, lactate; B, $+10^{-6}$ *M* FAD; C, $+10^{-5}$ *M* FAD.

followed, FMN suppresses the reduction of cytochrome b_2, as shown in Fig. 4. These converse phenomena might be caused by a certain difference between both experimental scales, FAD gives a similar result to FMN, as shown in Fig. 5.

Comparison in Reductions of Yeast Cytochrome c and Cytochrome b_2 by Lactate in the Presence of the Yeast Lactic Dehydrogenase Preparation—The reduction of cytochrome b_2 by lactate and by malate in the presence of the enzyme preparation are remarkably slower than those of yeast cytochrome c. As shown in Fig. 6, the reduction of cytochrome b_2 by an enzyme solution is almost same in rate as the reduction of yeast cytochrome c by the enzyme solution diluted forty two times. It is natural to consider that the reduction

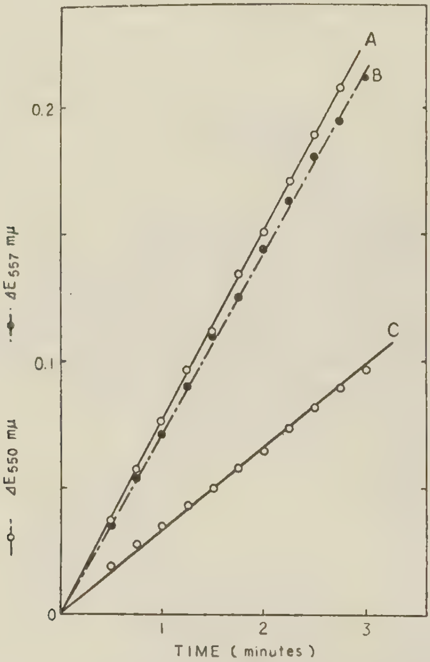


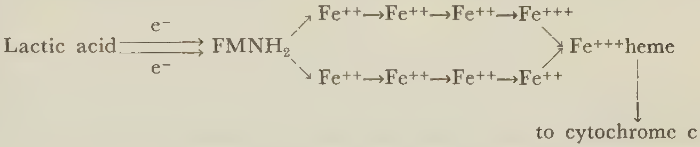
Fig. 6. Comparison in reductions of cytochrome c and of cytochrome b_2 by lactate in the presence of yeast lactic dehydrogenase preparation.

The components of reaction mixture were as follow; 0.1 *M* phosphate buffer of pH 6.4, 0.1 *M* sodium lactate of pH 6.4, the enzyme solution, baker's yeast cytochrome c or cytochrome b_2, in 4.0 ml. of the total volume. Reactions were carried out in nitrogen gas at 18°. Curve A, reduction of cytochrome c by the enzyme solution diluted 42 times as much as a standard enzyme solution; B, reduction of cytochrome b_2 by the standard enzyme solution; C, reduction of cytochrome c by the enzyme solution diluted 63 times.

of the cytochrome b_2 fixed to the yeast latic dehydrogenase in a certain conjugate form must be faster than that of the cytochrome c existing apart from the dehydrogenase-complex. Therefore, this extremely slow reduction of the cytochrome b_2 added in an isolated form may indicate the remarkable decrease in efficiency resulted from splitting the cytochrome b_2 moiety from the other moieties of yeast lactic dehydrogenase.

DISCUSSION

Baker's yeast lactic dehydrogenase has beed recently crystallized by Appleby and Morton (*1*). The crystals contain FMN, show the absorption spectra of so-called cytochrome b_2 and catalyze the reduction of cytochrome c by lactate without addition of coenzyme. Boeri, Tosi *et al.* (*8*, *9*) have found that the dehydrogenase contains one FMN residue, one heme group and eight iron atoms not bound to heme for each 230,000 g. protein, and they demonstrated the absence of a diphosphopyridine nucleotide-dependence of the dehydrogenase activity, using their best preparation of the dehydrogenase. Then, they have proposed the following scheme (*9*) for the electron transport in the enzyme;



Recently we have succeeded in crystallization of cytochrome b_2 from baker's yeast. The cytochrome b_2 contains no flavin and does not show any enzymic activity, indicating that the crystalline cytochrome b_2 corresponds to the part visualized as "Fe^{+++} heme" in the above scheme. On the other hand, Singer, Kearney *et al.* (*10*, *11*) have purified succinic dehydrogenase from bovine heart mitochondria in a homogeneous state and from bake's yeast in 60 per cent of purity. This water-soluble dehydrogenase has been found to contain 4 atoms of ferrous (non-heme) iron and a mole of flavin dinucleotide per mole of protein (200,000 g. in the case of the mammalian enzyme) but not any heme. On the other hand, cytochrome b has been highly purified with help of cholate from bovine heart muscle by Sekuzu and Okunuki (*12*). The cytochrome b does not show succinic dehydrogenase activity, and it is remarkably autoxidizable as well as cytochrome b_2. All of the schemes proposed for the electron-transferring system in mammalian mitochondria show that succinic dehydrogenase catalyzes the electron transport from succinate to cytochrome b. However, the succinic dehydrogenase highly purified in a water-soluble state does not catalyze the reduction of cytochrome b solubilized with help of cholate by succinate (*13*). When cytochrome b_2 is used as the final electron acceptor from lactate instead of cytochrome c, yeast lactic dehydrogenase can reduce cytochrome b_2 only in an extremely slower rate than the cytochrome b_2

contained in the yeast lactic dehydrogenase preparation or cytochrome c. This fact suggests an importance of a conjugate form of the dehydrogenase moiety, the flavin moiety and the cytochrome b_2 moiety in displaying fully the catalytic action from lactate to cytochrome b_2. The relation between succinic dehydrogenase and cytochrome b might be similar to that between the lactic dehydrogenase-flavin moiety and the cytochrome b_2 moiety of yeast lactic dehydrogenase.

A simple visualization for yeast lactic dehydrogenase may be as follows; substrate→[dehydrogenase FMN cytochrome b_2]→cytochrome c, where "dehydrogenase" exists as a protein unit as well as "cytochrome b_2" and both of them form a certain conjugate form together with FMN. At a lower or higher pH, the binding of these moieties of yeast lactic dehydrogenase might be split between the dehydrogenase-FMN moiety and the cytochrome b_2 moiety, since the reductions of cytochrome b_2 by lactate and by malate in the presence of the yeast lactic dehydrogenase hardly decrease in rate at pH 4.0 and pH 9.0, while the reductions of cytochrome c remarkably descrease, and moreover, cytochrome b_2 is much more extracted at pH 8.5 from the baker's yeast cells disruped by ethylacetate-treatment than at a lower pH (*6*).

SUMMARY

The role of action of the cytochryme b_2 contained in baker's yeast lactic dehydrogenase was re-evaluated by using the cytochrome b_2 separated from the dehydrogenase as the final electron acceptor.

1. Cytochrome b_2 is reduced anaerobically by lactate and by malate in the presence of the yeast lactic dehydrogenase preparation. The reduction by malate is much slow in rate compared with that by lactate as well as in the case where yeast cytochrome c is used as the final electron acceptor.
2. Cytochrome b_2 is notably autoxidizable.
3. The reductions of cytochrome b_2 by lactate and by malate in the presence of the enzyme preparation are different in optimum pH from those of cytochrome c by both substrates. In the latter cases, the optima pH are at 6.5 and 8.0, while in the former cases, the reductions by lactate and by malate hardly decrease in rate at pH 4.0 and 9.0, respectively.
4. The reduction of cytochrome b_2 by lactate in the presence of the enzyme preparation is partially inhibited in the presence of FMN or FAD, when it is measured spectrophotometrically.
5. The reduction of cytochrome b_2 by lactate in the presence of a test solution of yeast lactic dehydrogenase is almost same in rate as the reduction of cytochrome c in the presence of the test solution diluted forty two times.

Based upon these facts, the role of action of cytochrome b_2 in the dehydrogenations of lactate and of malate in the respiring yeast cells was discussed with stress on an importance of a conjugate form between the

cytochrome b_2 moiety and the dehydrogenase-FMN moiety in yeast lactic dehydrogenase.

We are very grateful to Mr. K. Fujii of Oriental Yeast Co., Ltd., Osaka for supplies of baker's yeast. We should like to thank our colleagues Messrs. M. Nozaki, T. Higashi, H. Matsubara, T. Yamanaka and H. Mizushima for helpful discussions during the course of this work.

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The Journal of Biochemistry, Vol. 45, No. 9, 1958

DENATURATION OF MYOSIN B

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As is well known, the functions of myosin B are destroyed by prolonged incubation at high temperature, as usually observed in the proteins. As far as we know, few reports have been contributed to studies on the denaturation of myosin B. Blum (*1*) observed the disaggregation of myosin B at high pH value; Ouellet *et al.* (*2*) studied on the thermal deactivation of the adenosine triphosphatase (ATPase) of myosin B in KCl solution containing 0.09 g. myosin B/litre.

Recently, the investigation of the change in physico-chemical properties of this protein during the storage period has become an urgent need, in connection with the physico-chemical properties of mysin B (*3*) and the changes of muscle structural proteins through the processes of sausage manufacturing (*4*). Therefore, the denaturation of myosin B was investigated in some detail within a wide range of pH (5.8-8.9) and temperature (0–30°). It was found that the denaturation consists of two steps, viz., a rapid first-order reaction independent of pH and a slow one greatly influenced by the pH values.

MATERIALS AND PROCEDURE

Materials—Myosin B (natural actomyosin) was prepared by extracting rabbit muscle for 24 hours with the Weber-Edsall solution and purified by repeating the usual "dilution-precipitation method" two or three times.

Myosin was prepared from rabbit muscle according to the method described by Perry (*5*).

Adenosine triphosphate (ATP) was isolated after the method of Kerr (*6*) from alcohol-treated fresh rabbit muscle and was used as the potassium salt. The concentration was determined from ultraviolet absorption at 260 mμ by means of a Hitachi spectrophotometer ($E_{1cm}=14{,}900$).

Procedure—Nine ml. 0.6 *M* KCl solution of myosin B, containing adequate concentration of buffer reagents and 0.01 *M* $CaCl_2$, was incubated in a constant temperature bath at 20° for five minutes, and then 1 ml. of 0.01 *M* ATP solution was added to the enzyme solution, thus starting the reaction. At measured intervals of time, aliquotes of the reaction mixture were pipetted into equal volume of 6 per cent trichloroacetic acid or 10 per cent perchloric acid. The precipitated protein was eliminated by filteration through a dry filter. Phosphate liberated was measured colorimetrically, using the Fiske-SubbaRow method. Enzyme assays were carried out at pH 5.9, 6.8, 7.3 or 8.9, tris-maleate buffer (pH 5.9–7.3) or glysine-KOH one (pH 8.9) being used.

The change of viscosity of myosin B after the addition of ATP was determined in 0.6 *M* KCl and 10^{-3} *M* $MgCl_2$ solution at pH 6.8 and 20°, using a viscosimeter of the Ostwald type.

Salting-out analysis was made employing the technique of Derrien (*7*). Experimental conditions were almost the same as those under which Snellman and Tenow (*8*) carried on their work. Ammonium sulfate solution in the various degree of saturation was prepared. To 5 ml. of this solution was added 1 ml. of protein solution (0.6 *M* KCl) and the mixture was allowed to stand at 1° for 20 hours. Then protein solutions were filtered and the protein concentration of the filtrate was measured by Folin's phenol reagent (*9*) with careful attention to the inhibitory action of excess ammonium sulfate.

Ultracentrifugal analysis was made by means of a Spinco model E ultracentrifuge at 59,800 r.p.m., at 28°. The protein content was calculated by multiplying the nitrogen content determined by the micro-Kjeldahl method by a factor 6 (*10*). The pH was determined by a glass electrode pH meter.

The storage conditions of myosin or myosin B were as follows:

(I) The sample solution (0.6 *M* KCl) of myosin or myosin B was divided into two portions. One of them was stored as it was, using the protein as a buffer (pH about 6.5) and the other was stored at pH 5.8 secured by adding dil. lactic acid to the former. Both portions were stored in a refrigerator at 3°±1° and in an incubator at 20°±1°, respectively. Their enzymic and physico-chemical properties were examined at regular intervals of time. ATPase activity was measured at pH 6.8 in the presence of 0.01 *M* $CaCl_2$.

(II) Sample solution (0.6 *M* KCl) of the enzyme was divided into three portions, their pH being adjusted to 5.9, 7.3 and 8.9, respectively. They were kept in water baths at 1°±1°, 20°±0.5° and 30°±0.5°, respectively. The ATPase activities were assayed at regular intervals of time at the same pH as in the storage solution and in the presence of 0.01 *M* $CaCl_2$.

RESULTS

I. Myosin B-ATPase

Effect of Temperature—The deactivation of myosin B-ATPase was studied, following the procedure (I) and (II) described above, and the results are given in Fig. 1.

It is evident from this figure that the deactivation rate of myosin B depended remarkably upon the temperature as seen usually in case of the enzymes. However, the most significant fact in the present case was that the denaturation of myosin B consisted of two reaction stages; the first-order reaction at high velocity followed by a slow denaturation superimposed upon the former. The first-order rate constant of deactivation, k_D* calculated, for example, to be 0.012×10^{-4}. sec.$^{-1}$, at pH 5.8 and 20° (Fig. 1, curve, ×—×).

Effect of pH—The pH dependence of the denaturation rate of myosin B was examined under storage condition (II) and at 30°, and the results obtained are shown in Fig. 2. It was found more clearly than in the former experiment that there were two reaction steps, the first-order deactivation and the slow one, when the deactivation was observed within a wide range of pH values. The first-order deactivation constants k_D were found to be independent of pH at least in the pH range here used, their values at 1°, 20° and 30° being $<0.001\times 10^{-4}$, 0.02×10^{-4} and 0.7×10^{-4}. sec.$^{-1}$, respectively. The apparent activation energy, E, calculated from these value was about 57 Kcal./mole, that is, remarkably high as is usually seen in protein denaturation. However, the second slow reaction step was effected considerably by pH, particularly in an alkaline region.

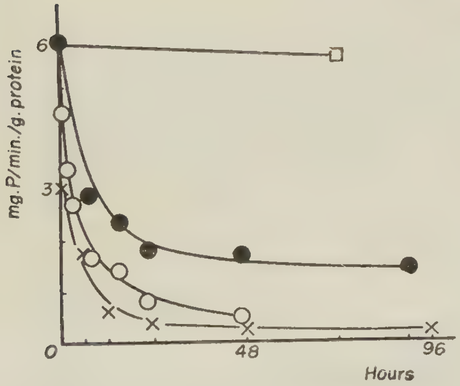

FIG. 1. Effect of temperature on the deactivation of myosin B-ATPase. —□—, 1°; —×—, 20°; —○—, 30°. pH 5.9 (tris-maleate buffer), 5.5 mg. of protein/ml. —●—, 20°. pH 5.8 (dil. lactic acid), 9.6 mg. of protein/ml.

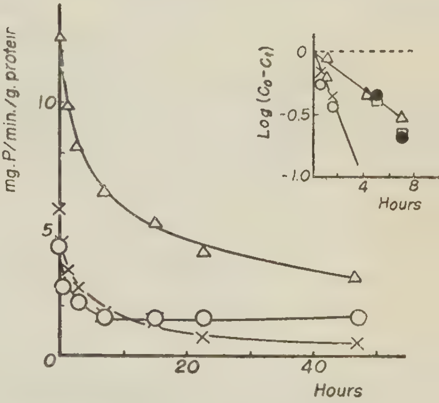

FIG. 2. Effect of pH on the deactivation of myosin B-ATPase. —△—, pH 8.9, glycine-KOH buffer; —×—, pH 7.3, Tris-maleate buffer; —○—, pH 5.9, Tris-maleate buffer. 5.5 mg. of protein/ml., 30°. The upper figure is inserted to show the first-order deactivation rate at various storage temperatures. ———, 1°; —△—, —●—, —□—, 20°; —×—, —△—, —○—, 30°.

Kinetic Analysis—As is well known, the relation between the reaction velocity of myosin B-ATPase and the concentration of ATP is given by the Michaelis-Menten formula (*2*, *11*),

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* The first-order deactivation rate constant k_D is calculated from the following formula, $k_D=\frac{1}{t}(\ln C_o-\ln C_t)=\frac{1}{t}\ln\frac{C_o}{C_t}$ where k_D is the first-order rate constant of deactivation, $\ln C_o$ and $\ln C_t$ are the logarithms, respectively, of the ATPase activity of myosin B solution (0.6 *M* KCl) before and after adequate incubation time (t).

$$V=\frac{V_{\max}}{1+\frac{K_m}{(\mathrm{ATP})}}$$

where V is the reaction velocity of ATPase, $V_{\max}$ is the reaction velocity at sufficiently high concentration of the substrate, K_m is Michaelis constant and (ATP) is the concentration of ATP. Then, myosin B solution (0.6 M KCl, pH 7.3) was stored at 20° under condition (II) and K_m and $V_{\max}$ were measured before and after 48 hours' storage. As tabulated in Table I, it was observed that $V_{\max}$ decreased considerably with storage, whereas K_m remained almost constant.

TABLE I

Michaelis Constant and $V_{\max}$ of Myosin B-ATPase before and after 48 Hours' Storage at 20°

Incubation time (hours)	0	48
K_m (M/litre)	2.2×10^{-4}	2.1×10^{-4}
$V_{\max}$ (*mg.* P/*min.*/*g.* protein)	4.96	1.80

II. Myosin-ATPase

The 0.6 M KCl solution of myosin was investigated as to its thermal deactivation of ATPase under storage condition (I). As shown in Fig. 3, the deactivation course of myosin-ATPase was similar to that of myosin B.

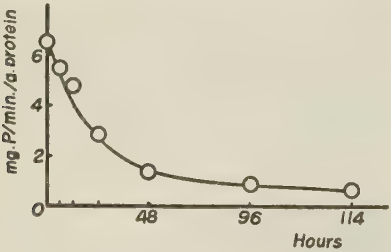

FIG. 3. Effect of temperature on the deactivation of myosin-ATPase. Initial pH 6.2, 20°, 8.0 mg. of protein/ml., 0.6 M KCl.

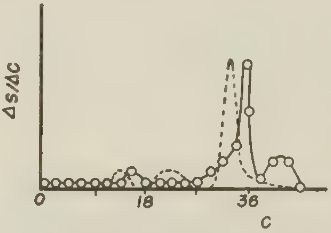

FIG. 4. The derived salting-out curve. S the extinction at 750 mμ, C=per cent (V/V) of saturated ammonium sulfate solution. The solid line and the dotted one represent, respectively, the curve before and after storage at 20°, pH 5.8.

III. Salting-out Analysis

Salting-out analysis by ammonium sulfate was carried out in order to elucidate the change of the fractions of myosin B during storage (I) for 48 hours. In Fig. 4, the differential extinction ($\Delta S/\Delta C$) is plotted against the concentration of ammonium sulfate. As is well established by previous workers (*12–14*), the ranges of the concentration of ammonium sulfate for the precipitation of the series of the principal structure proteins are as follows: actin (9–20 per cent), actomyosin (28–32 per cent) and myosin (33–45 per cent). Before storage, the salting-out peaks of myosin B showed the typical form, while the peak of myosin disappeared almost completely after 48 hours' storage, and further changes could not be observed in the figure, even when the storage time was longer than 48 hours. This change of the solubility curve remained the same in the pH range from 5.8 to 6.5.

IV. Ultracentrifugal Sedimentation

Ultracentrifugal runs of 0.5 *M* KCl solution of myosin were performed before and after about 40 hours' storage under condition (II). As shown in Fig. 5, the original single peak of myosin split into two peaks and the main peak spread to some extent.

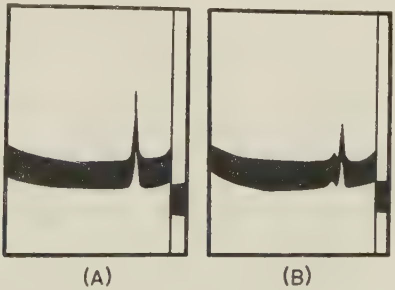

FIG. 5. Ultracentrifuge runs on native and denatured myosin (59,800 r.p.m., at 28°).

(A) Native myosin. (B) Denatured myosin (20°, 48 hours' storage). 2.0 mg. of protein/ml., pH 6.5, 0.5 *M* KCl, after 32 minutes.

V. The Change of Viscosity

The ATP sensitivity is defined as follows (*15*):

$$\text{ATP sensitivity} = \frac{Z\eta_{\text{rel}} - Z\eta_{\text{ATP}}}{Z\eta_{\text{ATP}}} \times 100$$

where $Z\eta_{\text{rel}}$ and $Z\eta_{\text{ATP}}$ represent the logarithms, respectively, of the relative viscosities of myosin B solution before and after the addition of ATP.

The change of ATP sensitivities of myosin B during storage (I) coincided completely with that of ATPase under the same condition (Fig. 6). However, for the estimation of ATP sensitivity after long storage, it was necessary to eliminate denatured aggregates which were formed with the development of the denaturation, since they cosiderably changed the relative viscosity.

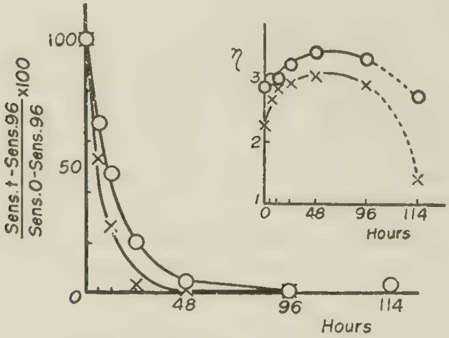

FIG. 6. Change of ATP sensitivity during storage of myosin B. ○, initial pH 6.5; ×, initial pH 5.8. 20°, 9.6 mg. of protein/ml. Sens. O; ATP sensitivity of native myosin B, Sens. 96; ATP sensitivity of myosin B after 96 hours' storage, Sens. t; ATP sensitivity of myosin B after fixed hours' storage. The upper figure shows the change of relative viscosity (η).

DISCUSSION

As described above, the denaturation of myosin B was found to consist of two reaction stages; the first rapid reaction of the first-order and the second slow but complete denaturation which occurred in superposition upon the former. The rates of the first reaction were observed to be independent of pH in the range from 5.8 to 8.9 and, noticeably, to destroy the enzymic activity partially but not completely (about 70–85 per cent). The heat of activation (57 Kcal-/mole) of the deactivation of myosin B-ATPase reported in this article was somewhat lower than that reported by Ouellet *et al.* (70 Kcal/mole). Although the cause of this difference between two values was not clarified, it might be due to the difference in the method of extracting the protein and in the protein concentration in the storage solution.

As the ATPase activity of myosin B is attributed to the myosin component, the change in myosin B-ATPase may originate in the structural change of myosin. In fact, the deactivation of myosin-ATPase nearly coincided with that of myosin B, and the structural changes in the myosin component were clearly shown from the salting-out and from the ultracentrifugal analysis. Furthermore, the change in the ATP sensitivity of myosin B coincided with that of the deactivation of ATPase. It can, therefore, be concluded that the denaturation of the myosin component dominates the one of myosin B.

The following alternatives may be possible as the mechanism of the denaturation; the one is that in which all myosin molecules are denatured uniformly, and the other is that in which some molecules of myosin are denatured completely, while others keep their original properties. As mentioned above, K_m remained constant, even when $V_{\max}$ decreased remarkably. Consequently, it seems conceivable that the latter mechanism offers an explanation preferably to the former.

Since the denaturation of actomyosin seems to play an important role in the change of the binding quality of sausage, the results stated above may be important not only in the fundamental chemical study of myosin B (*3*) but also in practical food research (*4*). The latter aspect will be described in forthcoming paper.

SUMMARY

1. The deactivation of myosin B-ATPase during storage consisted of two steps: the first pH-independent, a rapid one of the first-order, and the second pH dependent, a slow one. The activity was destroyed partially by the former reaction; complete denaturation occurred only when both of two steps occurred. The first-order rate constants of the first one were 0.70×10^{-4} and 0.02×10^{-4}. sec.^{-1} at 30° and 20°, respectively, and the activation energy was about 57 Kcal./mole. The second reaction was slow at neutral pH value but was remarkably fast in the alkaline region.

2. The Michaelis constant of ATPase remained almost constant during the denaturation of the protein.

3. The deactivation of myosin proceeded rather similarly with that of myosin B.

4. In the salting-out curve, the peak of the myosin fraction disappeared as the denaturation of myosin B proceeded.

5. When myosin denatured, its ultracentrifugal pattern split into two peaks and the main one spread to some extent.

6. The change of ATP sensitivity during storage coincided completely with that of ATPase under the same condition.

The authors would like to express their cordial thanks to Dr. Y. Tonomura of the Institute for Catalysis for his warm-hearted support and valuable advice during the course of the work and the preparation of the manuscript and also to Mr. Nihei of the Low Temperature Institute of this University for his help in kinetic analysis. This study has been aided in part by a grant in aid from the Scientific Found of the Ministry of Education to the Research Group on the "Protein Chemistry of Meat Products".

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The Journal of Biochemistry, Vol. 45, No. 9, 1958

CRYSTALLINE CYTOCHROME C

III. CRYSTALLIZATION OF FISH CYTOCHROME C AND A THIRD METHOD OF PURIFICATION*

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(Received for publication, February 27, 1958)

In preceding papers of this series (*1*, *2*) two methods were reported which permitted the crystallization of cytochrome c from mammalian hearts and pigeon breast muscle. The first method, designed as Method I and described in Paper I (*1*), involved the precipitation of the cytochrome with TCA** in the presence of AS**, while in the second one (Method II), which was detailed in Paper II (*2*), the use of TCA was avoided and the pigment was directly absorbed onto the resin Amberlite XE-64 from acidic tissue extracts containing high concentrations of AS. Both methods were equally applicable for preparing crystalline products from the mammalian as well as avian sources, but the second method appeared to be somewhat superior to the first one in causing less modifications of the pigment protein.

Cytochrome c of fish origin was for the first time obtained by Paléus (*3*) in a highly purified state from salmon heart. However, we were the first to succeed in crystallizing the pigment from fish tissues (*4*); the starting materials used were bonito and tunny hearts and the method employed was essentially the same as Method I except that the cytochrome was extracted from tissues without acid treatment.

In the present paper we wish to report that the fish cytochrome c can be crystallized not only by Method I but also by Method II and by a third method in which the cytochrome was extracted at neutral reactions from tissues pre-treated with acid and then directly adsorbed onto the resin. This newly introduced method, Method III, has been found most suitable for purification and crystallization of cytochrome c from beef heart and pigeon breast muscle.

EXPERIMENTAL

Bonito and tunny hearts were sent to the laboratory in frozen state. They were freed from arterial bulbs, washed with small amounts of water, and then minced in partly frozed states. Fresh beef heart and pigeon breast muscle used were minced as described before (*1*,*2*).

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* Priliminary report of this paper was communicated in the "*Proc. Japan Acad.*, **34**, 169 (1958)

** Trichloroacetic acid and ammonium sulfate are abbreviated in this paper as TCA and AS, respectively.

The resin Amberlite XE-64 was treated as previously reported (*1*, *2*), and the cytochrome was assayed by the same method as described in Paper I (*1*).

Purification and Crystallization—Methods I and II were described in Papers I and II, respectively.

Details of Method III were as follows:

Step 1. Extraction: One kg. of minced fish hearts was treated with 400 ml. of 1 *N* acetic acid at 5° for 1 hour as described for Method II (*2*), and 600 ml. of 0.1 *N* ammonia were added with vigorous stirring. After standing for a few minutes, the mixture was brought to pH 5.6 to 6.0 by addition of 30 per cent ammonia and kept at room temperature (about 20°) for 3 hours or in the cold (about 5°) overnight. The suspension was well mixed with 150 g. of coarse celite (Celite 545, Tons-Manville Co.) and squeezed out through cloth with the aid of a press. The residue was resuspended in 500 ml. of water and, after standing at room temperature for 1 hour, squeezed out again. The combined extracts were adjusted to pH 6.0 to 6.2 with 30 per cent ammonia, filtered through a Buchner funnel with the aid of 30 g. of Hyflo Super-Cel, and the residue was washed with 200 ml. of water. The filtrate and washing were combined. Clear brown solutions were obtained from fish and beef hearts, and a colloidal brown solution from pigeon breast muscle. About 2,000 ml. in volume.

Step 2. First Adsorption and Elution: The extract obtained from Step 1 was passed through a bed of Amberlite XE-64 (6 cm. in diameter, 4 cm. high) placed in a glass filter. Prior to the use, the resin was usually equilibrated with ammonium phosphate buffer of pH 6.0. It was, however, found that the resin equilibrated at pH 7.0 was also satisfactory if it was used after washing with a small amount of water. The resin which had adsorbed cytochrome c was washed with 200 ml. of 0.05 *N* ammonium phosphate buffer of pH 7.0. The liquid in the bed was removed by sucking and the upper part of the bed, colored in dark red, was carefully separated with a spatula from the colorless part. The colored resin was then suspended in about 500 ml. of the 0.05 *N* buffer, and the suspension was adjusted to pH 7.5 by dropwise addition of 30 per cent ammonia with stirring. The neutralized resin suspension was slowly poured on a layer of fresh resin, equilibrated at pH 7.0 and about 1 cm. high, which was placed in a glass filter of 4 cm. in diameter. The whole resin was washed by passing about 500 ml. of 0.1 *N* buffer pH 7.0 (0.125 *N* in the case of pigeon muscle). Cytochrome c was then eluted by slowly passing 0.5 *N* buffer of pH 7.0 through the resin bed and the reddish effluent solution was collected as described for Step 3 in Method II (*2*). Reddish effluent solution. About 50 ml., containing about 0.4 *N* NH_4^+, pH 6.5.

Step 3. Second Adsorption and Elution: Exactly the same procedure was employed as Step 4 in Method II (*2*).

Step 4. Chromatography: The procedure was the same as Step 5 in Method I (*1*), except that the amount of resin required for tunny cytochrome c was about twice as much as that used for the cytochrome from other tissues.

Step 5. Crystallization: This was the same as Step 6 in Method I (*1*).

Crystallization of Fish Cytochrome c in Oxidized Form—The oxidized form of fish cytochrome c could be crystallized according to essentially the same procedure as reported for the pigeon cytochrome c (*2*), except that impurities were removed at pH 8 to 9 from the chromatographically concentrated solution of cytochrome c by adding 0.4 g. of AS per g. of solution. This condition was chosen in view of the slightly insoluble nature of the fish pigment as compared with pigeon cytochrome c. The solution freed from impurities was allowed to stand overnight at room temperature (15 to 20°) so that it became neutral due to the evaporation of ammonia. Powdered AS was then added until the solution became slightly turbid. The oxidized form of the fish cytochrome c seems to be more easily crystallizable at pH 5.5 to 7.5 than at higher pH's. It was recently found that beef heart cytochrome c purified according to Method III is also crystallizable in oxidized form at pH 5.5 to 6.5.

RESULTS AND DISCUSSION

Extraction—Sulfuric acid was first used by Theorell (*5*) as an extracting reagent of cytochrome c from mammalian hearts. Keilin and Hartree (*6*) later introduced TCA which is now widely used for this purpose. Recently, sulfuric acid was again used for the extraction of the cytochrome from salmon heart (*3*).

In previous papers (*2, 7*) we reported that cytochrome c could be extracted from mammalian and fish tissues with concentrated AS solutions even at neutrality and found that this procedure caused less modification of the cytochrome protein. In this method, however, the pigment could not be sufficiently extracted and therefore repeated extractions were required.

In order to avoid this difficulty the procedure has since been improved in such a way that the minced muscle was treated with acetic acid prior to the AS extraction (*1*, *2*). Recently, we have further found that the cytochrome can be effectively extracted at neutral pH's even in the absence of added AS if the tissues pre-treated with acetic acid are used. This last mentioned procedure has therefore been incorporated into Method III.

A defect of this new procedure lies in the fact that it results in the extraction of rather large amount of impurities. This disadvantage can, however, be more compensated by the fact that the cytochrome in such extracts can be directly adsorbed onto the neutral resin.

Table I shows a comparison of various extraction procedures. As can be seen, cytochrome c is readily and effectively extractable with water if the tissues are preliminary treated with acetic acid. It is apparent that such water extraction is a much milder procedure than the use of TCA or sulfuric acid. Squeezing process, however, becomes somewhat more difficult in this method. 100 to 150 g. of coarse Celite are therefore necessary to facilitate the process per kg. of mince.

Direct Adsorption of Cytochrome—Experiments C, G, H and I in Table I show that the extraction of cytochrome c with concentrated AS solutions is a quite excellent procedure in respects to yield, purity of extracted pigment, and easiness of squeezing. The cytochrome, however, cannot be directly adsorbed onto the neutral resin from such extracts due to the high concentrations of AS. In Method I (*1*), therefore, the cytochrome is precipitated with TCA prior to the resin treatment, while in Method II (*2*) the pigment is adsorbed onto the resin at acidic pH's where the resin also adsorbed large amounts of impurities.

If dilute TCA, sulfuric acid or acetic acid is used as an extracting

TABLE I

Comparison of Extraction Conditions

Source	Beef heart			Pigeon breast muscle			Tunny heart		
Extraction conditions	Amount of cyt. c *	Purity of cyt. c **	Easiness of squeezing	Amount of cyt. c *	Purity of cyt. c **	Easiness of squeezing	Amount of cyt. c *	Purity of cyt. c **	Easiness of squeezing
A. Water	+	+	++	+	+	++	+	+	+
B. AS, pH 7	++	++	++++	++	++	++++	++	++	++++
C. AS, pH 4.3	+++	++++	++++	++++	+++++	++++	++++	++++	++++
D. H_2SO_4, pH 4.2	+++	+++	++	++++	++	+	++++	++	++
E. TCA, pH 4.2	++++	++++	++++	++++	++++	++++	+++	++++	++++
F. HAc, pH 4.2	++++	++	++	++++	+	+	++++	++	+
G. HAc, pH 4.6 →AS, pH 6	++++	++	++++	++++	++	++++	++++	++	++++
H. HAc, pH 4.3 →AS, pH 6	++++	++	++++	++++	++	++++	++++	++	++++
I. HAc. pH 4.3 →AS, pH 4.8	++++	++++	++++	++++	++++	++++	++++	++++	++++
J. HAc, pH 4.3 →H_2O, pH 6	++++	++	++	++++	++	+	++++	++	++

Increasing number of the +sign indicates increase in the amount of extractable cytochrome c, purity, and easiness of squeezing. In each experiment the mince was extracted with the same volume of extracting reagent described below at 20° for 2 hours. Extracting reagents used were as follows: A, distilled water; B, 85 per cent saturated AS (finally 50–60 per cent AS saturation) adjusted to pH 7.0 with NH_4OH; C, 85 per cent saturated AS adjusted to pH 4.3 with glacial acetic acid; D, 0.12 to 0.14 *N* H_2SO_4 (pH 4.1 to 4.3); E, 0.13 to 0.15 *N* TCA (pH 4.1 to 4.3); F, 1.5 to 2.0 *N* acetic acid (pH 4.1 to 4.3); G, each g. of mince was treated with 0.4 ml. of 0.25 *N* acetic acid for 1 hour at 5°. To the suspension (pH 4.6) were added 0.55 ml. of 0.17 *N* NH_4OH and 0.65 g. of solid AS per g. of mince (finally *ca.* 55 per cent AS saturation, pH 6.0) and extracted for 2 hours. H, the same as G except that 0.5 *N* acetic acid was used (the pH of suspension, 4.3). I, Treated with 0.5 *N* acetic acid as in H and extracted without addition of NH_4OH (finally 55 per cent AS saturation, pH 4.8). J. treated with 0.5 *N* acetic acid as in H and extracted without addition of AS at pH 5.8 to 6.0.

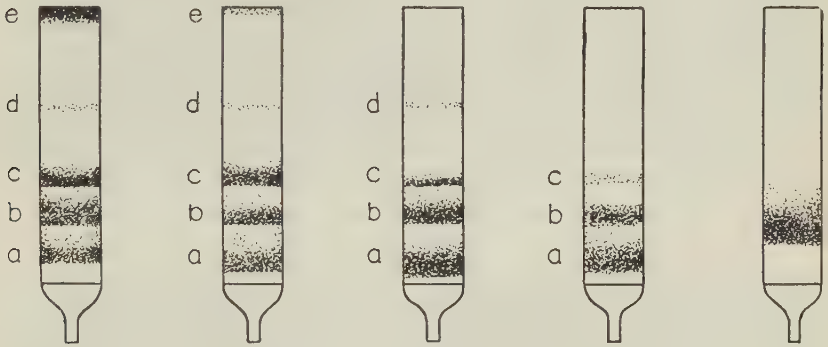
* Assayed according to the method described previously (*1*) after treating the extracts with AS (*ca.* 60 per cent saturation) at pH 4.2 to remove colored impurities, the results were only crude approximations. Cytochrome c was also estimated by a hand spectroscope.

** Estimated from the yield of cytochrome c and the amount of protein precipitable with TCA.

reagent (Table I, Experiments D, E, F), cytochrome c can easily be adsorbed onto the resin from the extracts at pH 5.5 to 7.0, a pH range where the cytochrome adsorbing capacity of the resin is much higher than below pH 5.

As shown in Experiment J in Table I, the cytochrome extracted at pH 6 from acetic acid treated mince without addition of AS can also be readily adsorbed on the resin at pH 5.5 to 6.5. If such an extract is diluted 1.5 to 2-fold water, the adsorption of cytochrome is complete even at higher pH's (*e.g.*, pH 7 to 7.5) where a large part of impurities passes through the resin bed. However, at such higher pH's a considerable part of pigment is converted to the reduced form presumably due to the presence of reducing substances which are more reactive at these pH values. This phenomenon makes it necessary to use a larger amount of resin because of the lower affinity of reduced cytochrome to resin than that of oxidized from.

It was, therefore, decided in Method III to extract the cytochrome at pH 5.6 to 6.0 and to carry out the resin adsorption at pH 6.0, a pH at which almost all the cytochrome is still in the oxidized from.



A
TCA-AS
method
unny heart)

B
Acid resin
method
(tunny)

C
Neutral resin
method
(tunny)

D
Same as C
(washed
mince)

E
Neutral resin
method
(beef)

FIG. 1. Comparison of chromatograms of cytochrome c samples obtained by various methods. Column; Amberlite XE-64. Buffer; 0.25 *N* ammonium phosphate, pH 7.0.

Modification of Cytochrome Protein—It was already reported (*1*, *2*) that cytochrome c from beef heart and pigeon breast muscle underwent considerable modifications during the TCA-AS precipitation procedure in Method I, whereas much less modifications took place in the direct absorption procedure in Method II. The direct adsorption, however, had to be carried out below pH 5.0 owing to the presence of large amounts of AS in the extracts. Such low pH values seemed to cause some modifications on the fish cytochrome, although their extents were much less than those due to the TCA-AS treatment (see Fig. 1 A and B). Such modifications were minimized in Method III by performing extraction in the absence of added AS and adsorption at nearly neutral reactions.

It was, however, found that the cytochrome obtained by this procedure was separated on chromatography into 3 bands (Bands a, b and c in Fig. 1), although the amounts of cytochrome in two minor bands were comparably smaller than when Method I or II was applied. This is in contrast to the cytochrome obtained from beef heart or pigeon muscle which usually gives only one band if the TCA treatment is avoided.

All these three hands yielded crystals of cytochrome c of the same purity and essentially the same shape. It is, therefore, difficult to decide whether some of these bands represent slightly modified cytochrome c or all of them are native pigments with slightly different properties. These three bands were also observed when the fish tissues were extracted at neutral pH's in the presence of AS and chromatographed after dialysis.

In one experiment tunny hearts were thoroughly washed with water before extraction. This procedure removed some of the cytochrome in the washing. The washed tissue was then treated according to Method III and the product was chromatographed. In the chromatogram obtained Band c was very slight and Band b also smaller. This fact seemed to indicate that the cytochrome components in Bands b and c might have been present in the original tissues in relatively water-soluble states (see Fig. 1, D).

In conclusion, however, it seems most probable that the pigments present in Bands b and c are not native ones; they might have been modified in hearts after their removal from fish. The hearts had probably been allowed to stand at room temperature for a considerable period of time before they were frozen. In fact, some of them were already beginning to autolyse.

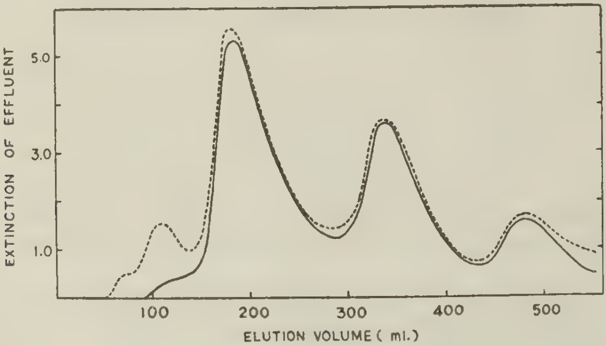
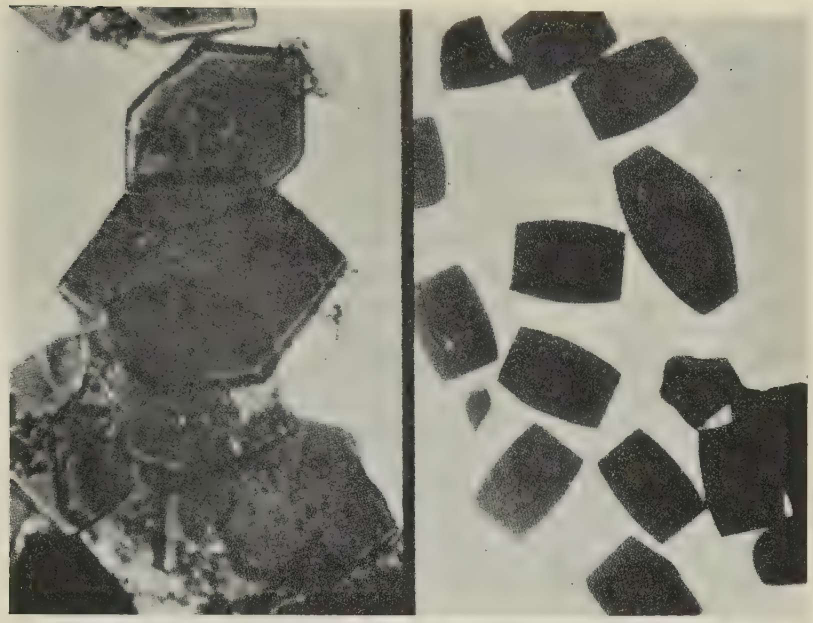


FIG. 2. Chromatogram of tunny heart cytochrome c. Cytochrome c preparation; Method III, Step 3. Column; Amberlite XE-64, (0.25 g. ion of NH_4^+), pH 7.0. Solid line; Extinction at 550 mμ, Brocken line; Extinction at 280 mμ.

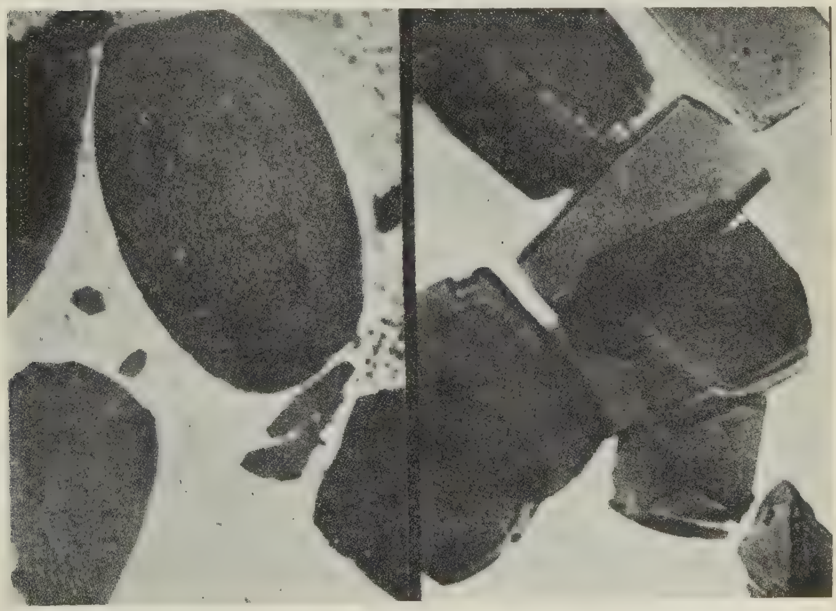
Chromatography—Fig. 2 shows a typical chromatogram of cytochrome c purified according to Method III from 1 kg. of tunny hearts. As can be seen, the pigment was separated into three fractions. Among them the last one was the smallest in amount. Essentially the same chromatograms were obtainable with bonito heart cytochrome c.

Crystals of Fish Cytochrome c—The reduced from of tunny and bonito



A. Usually obtainable plates (×300) B. Hexagonal plates (×400)

FIG. 3. Crystalline reduced cytochrome c of tunny heart.

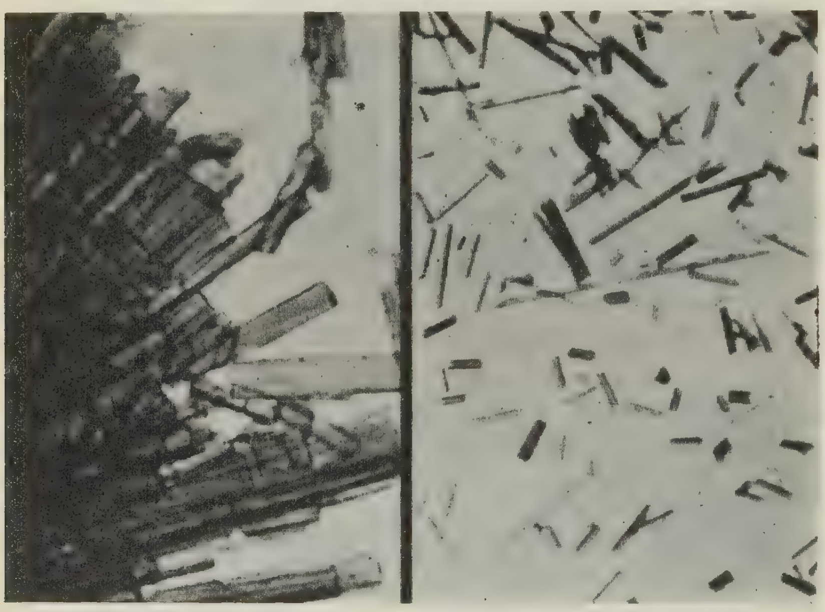


A. Usually obtainable plates (×300) B. Very large plates (×20)

FIG. 4. Crystalline reduced cytochrome c of bonito heart.

cytochrome c was easily crystallizable from any of the above three fractions. Crystals were usually obtained in plates as shown in Fig. 3, A. Sometimes regular hexagonal plates as shown in Fig. 3, B were observed. Square plates resembling those of the beef cytochrome were also occasionally obtained. These crystals were extraordinally large and sometimes reached a few mm. in size (see Fig. 4, B).

The oxidized form of fish cytochrome c was also obtained in crystalline state by a method similar to that employed for the oxidized form of pigeon cytochrome c. The crystals usually appeared as long prisms or plates as shown in Fig. 5, A and B.



A. From tunny heart (×30) B. From bonito heart (×150)

FIG. 5. Crystalline oxidized cytochrome c of the two fishes.

Comparison of Three Methods—Table II is a summary of the three methods of purification and crystallization of animal cytochrome c. Each of these methods has its own advantages as well as disadvantages.

The squeezing of original extracts is easiest in Method II and most difficult in Method III. Coarse filter aid such as Celite 545 is, therefore, required in Method III and even in Method I when pigeon breast muscle is used.

The amounts of cytochrome c extractable from tissues are practically the same in the three methods.

Precipitation of cytochrome c with TCA in the presence of AS (Method I) is most convenient to obtain highly purified product from a large amount of extract, but this procedure causes considerable modifications of

TABLE II

Summary of Three Crystallization Methods

Method I	Method II	Method III
Treat with acetic acid and extract with AS at pH 6.	Treat with acetic acid and extract with AS at pH 4.5.	Treat with acetic acid and extract with H_2O at pH 6.
Remove impurities with AS of sp. gr. 1.220.	Remove impurities with AS of sp. gr. 1.220.	
Precipitate with TCA at pH 4.5–5 in AS soln.	Adsorb on acidic resin at pH 4.5 in 50% sat. AS, wash well and elute.	Adsorb on neutral resin at pH 6, wash well and elute.
Adsorb on neutral resin, wash and elute.	Adsorb on neutral resin, wash carefully and elute.	
Chromatography of oxidized cytochrome c on resin using ammonium phosphate buffer of pH 7.0 and 0.25 g. ion of NH_4^+.		
Concentration by resin column and crystallization of reduced cytochrome c in nearly saturated AS at pH 8–9.		

TABLE III

Purification and Crystallization of Cytochrome c from Tunny and Bonito Hearts by Method II

Step	Tunny heart			Bonito heart		
	Volume (*ml.*)	Yield (*μ mole*)	Purity (E_{550}/E_{280})	Volume (*ml.*)	Yield (*μ mole*)	Purity (E_{550}/E_{280})
1. Extracts of 1kg. mince	1600			1650		
2. AS treatment	1800	31	0.04	1800	18	0.035
3. 1st adsorption	120	26	0.60	140	15	0.58
4. 2nd adsorption	25	23	0.82	16	12	0.75
5. Chromatography (total)		17.5			9.0	
(Fraction a)*	120	6.0	0.95	100	2.5	0.95
(Fraction b)*	230	4.0	1.00	180	5.0	0.97
(Fraction c)*	310	7.5	1.00	210	1.5	0.95
6. 1st crystallization (total)		13.0			6.5	
(Fraction a)*	1.0	4.5	1.02	0.5	2.0	1.02
(Fraction b)*	1.0	3.0	1.03	1.0	3.5	1.04
(Fraction c)*	2.0	5.5	1.03	0.5	1.0	

* See Fig. 1.

the cytochrome unless it is performed very rapidly in the cold. Adsorption of the pigment onto the acidic resin (Method II) is much milder. Nevertheless, a part of cytochrome c, especially that of fish heart, is converted in this process to modified forms. Adsorption of the cytochrome onto the neutral resin (Method III) is best in this respect. This procedure is, however, not applicable to extracts containing high concentrations of AS and, therefore, contaminant proteins cannot be removed by AS treatment prior to adsorption to the resin. The removal of impurities, therefore, should be done by very careful washing of the resin bed adsorbing cytochrome c.

In Method I in which the pigment is precipitated with TCA, only one resin adsorption and elution procedure is sufficient. In Methods II and III, on the other hand, this treatment should be repeated two times careful washing in order to obtain sufficiently purified cytochrome c for the subsequent chromatography.

TABLE IV

Purification and Crystallization of Cytochrome c from Tunny Bonito and Beef Hearts and Pigeon Breast Muscle by Method III

Step	Beef heart		Pigeon muscle		Tunny heart		Bonito heart	
	Yield (*μmole*)	Purity (E_{550}/E_{280})	Yield (*μmole*)	Purity (E_{550}/E_{280})	Yield (*μmole*)	Purity (E_{550}/E_{280})	Yield (*μmole*)	Purity (E_{550}/E_{280})
1. Extracts	?	0.01	?	0.01	?	0.01	?	0.01
2. 1st adsorp.	14.5	0.55	30.5	0.80	30.0	0.60	15.5	0.55
3. 2nd adsorp.	12.5	0.95	26.0	1.00	24.5	0.88	13.0	0.75
4. Chromato. (total)	10.5	1.30	22.5	1.18	18.5		10.5	
(Frac. a)*					8.5	0.98	3.5	0.93
(Frac. b)*					7.5	1.01	5.0	1.00
(Frac. c)*					2.5	1.00	2.0	0.98
5. 1st cryst.	9.0	1.30	18.5	1.20	14.5		8.0	
(Frac. a)*					6.5	1.02	2.5	1.05
(Frac. b)*					6.0	1.05	4.0	1.02
(Frac. c)*					2.0	1.02	1.5	1.05

* Only one cytochrome fraction was obtained on chromatography from beef heart and pigeon breast muscle.

Table III and IV show the yield and purity of cytochrome c at each step of the three methods using tunny, bonito, and beef hearts and pigeon breast muscle as starting materials. From these tables as well as from Table I and II in Paper II (*2*) it is apparent that the yield of crystalline cytochrome c is highest in Method III and lowest in Method I.

SUMMARY

A new method, designated as Method III, is described for purification and crystallization of cytochrome c from fish hearts. In this method the starting material is first treated with acetic acid and the cytochrome was extracted at about pH 6 in the absence of added salts. The pigment in the extracts is adsorbed to the resin Amberlite XE-64 equilibrated at pH 6.0 and then eluted after careful washing of the resin with a buffer of pH 7.0. The eluted cytochrome is further treated in similar ways to Methods I and II and crystallized as the reduced form. The yield of crystals is 14 μ moles from 1 kg. of tunny hearts and 8 μ moles from 1 kg. of bonito hearts.

The fish cytochrome can also be crystallized by Method II. Method III is, however, superior in causing less modifications. Fish cytochrome c can be crystallized in the oxidized form.

Method III is also very suitable for preparing crystalline cytochrome c from beef heart and pigeon breast muscle.

The authors wish to express their indebtness to Nagase Co., Ltd. for the supply of fish hearts and also to Messrs. T. Okishio and S. Urakabe of Medical School of this University for their assistance.

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The Journal of Biochemistry, Vol. 45, No. 9, 1958

STUDIES ON AMYLASE FORMATION BY BACILLUS SUBTILIS

VI. THE MECHANISM OF AMYLASE EXCRETION AND CELLULAR STRUCTURE OF BACILLUS SUBTILIS

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(Received for publication, March 4, 1954)

Many bacteria are known to produce extracellular enzymes, but the mechanism by which these high molecular substances get out the cell is totally unknown.

While we were studying the mechanism of amylase formation by *Bacillus subtilis*, it was found that when cells were grown aerobically in shaking culture and then left without further aeration, a very rapid autolysis occured (*1*). This phenomenon was called "anaerobic lysis" and was pronounced only in the phase of active amylase production. When cells are cultivated in media which are suitable for good growth but not for the production of amylase this phenomenon is not significant. Therefore, both phenomena, amylase production and anaerobic lysis seem to be intimately connected with each other. In subsequent studies an autolytic enzyme (called "autolysin") was isolated from the lysate (*2*) and its site of action was clearly established to be the cell wall (*3*, *4*).

These earlier results seemed to suggest the participation of autolytic enzyme in the excretion of amylase protein by this bacterium. Further studies were undertaken in order to get more knowledge on this point and on the nature of the mechanism regulating enzyme excretion.

From the results obtained together with those of earlier studies, a hypothesis is advanced on the mechanism of enzyme excretion.

METHODS

B. subtilis strain H, which was used throughout the previous studies, was also used in this investigation. The composition of the media is as follows: Soy medium; soluble starch 8 per cent, Na citrate 0.04 *M*, $(NH_4)_2HPO_4$ 0.15 *M*, KCl 0.02 *M*, $MgSO_47H_2O$ 0.002 *M*, $CaCl_2$ 0.001 *M*, ethyl alcohol 1 per cent (*v*/*v*), soy bean extract (each liter of medium contains the extract obtained by boiling 50 g. of soy bean with about 250 ml. of 0.1 per cent NaOH for 1 hour), pH 7.2. LBY medium; lactose 1 per cent, peptone 1 per cent, meat extract 0.5 per cent, yeast extract 0.2 per cent, NaCl 0.2 per cent, phosphate *M*/30, pH 7.2. LBY (E, 7.5) and LBY (E, 10) medium; LBY medium fortified with 7.5

per cent and 10 per cent of polyethylene glycol (molecular weight 400) respectively. Salt solution C ; $Na_2HPO_4 12H_2O$ 0.225 *M*, KCl 0.03 *M*, $MgSO_4 7H_2O$ 0.003 *M*, $CaCl_2$ 0.0015 *M*, pH 7.2.

Lysis was followed by measuring the optical density (O.D.) at 600 mμ in a spectrophotometer or by readings in a nephelometer. Oxygen uptake of cells was measured at 30° in the Warburg respirometer.

RESULTS

Effect of Cyanide (CN^-) *and 2,4-Dinitrophenol* (*DNP*) *on the Culture under Aerobic Condition*—Previous studies indicated that the autolytic enzyme, which acts on the cell wall participates in anaerobic lysis. However, the trigger mechanism by which anaerobiosis induces the action of this enzyme is still unknown. The following possibilities can be considered : (a) a reducing condition due to oxygen deficiency induces *action* of the autolytic enzyme, (b) lack of an adequate energy supply due to oxygen deficiency induces *action* of the autolytic enzyme, and (c) a reducing condition or lack of energy induces the *formation* of the autolytic enzyme. Inhibition experiments were performed to decide which is the most probable process.

Various known inhibitors were added to the shaking culture in the phase of active amylase production. One part of each of these cultures was shaken further, the other part was left without further aeration and turbidity of both cultures was followed. Thus the ability of various inhibitors to replace the " anaerobic condition " and their effect on the " anaerobic lysis " itself were examined simultaneously.

From the data summarized in Table I, the following facts can be

TABLE I

The Effect of Various Inhibitors on the Lysis of a Bacillus subtilis Culture in the Phase of Active Amylase Production

Conditions	Expt. No.	Time of Incubation (*hr.*)	" Per cent lysis of culture "						
			No. Addition	CN 0.01 *M*	Azide 0.01 *M*	Azide 8×10^{-4} *M*	DNP 8×10^{-4} *M*	CN 0.01 *M*)+DNP 8×10^{-4} *M*	Chloramphenicol 100 μg./ml.
Anaerobic	1	1.0	31	30	70		58		28
	2	2.0	38	36		55			
Aerobic	1	2.5	2	44	83		14		6
	2	3.0	6	27		9.5		60	

Cells of *B. subtilis* H were cultivated in Soy medium for 22 hours at 30° with shaking. 1.5 ml. of the various inhibitor solutions were added to 5 ml. of the culture and either shaken further (aerobic) or left in a narrow test tube without further aeration (anaerobic). The O.D. of the culture was followed and the " per cent lysis " was calculated from the ratio of the decrease of O.D. to the initial O.D.. Final concentration of inhibitor is shown in the table.

recognized.

1. CN^- (0.01 M) which inhibited the respiration of this bacterium (Fig. 1) caused lysis even under the aerobic condition, but not exerted any stimulating effect on anaerobic lysis.

2. DNP, which was shown to inhibit oxidative phosphorylation of this bacterium greatly at a concentration of $1 \times 10^{-4}\ M$ (*5*) exerted little lytic effect even at a much higher concentration ($8 \times 10^{-4}\ M$) on the aerobic culture. However, DNP greatly stimulated anaerobic lysis. It was concluded that the lack of adequate energy supply *per se* does not seem to be a primary cause of anaerobic lysis, but accelerates the lysis once the trigger mechanism has induced the lysis of culture. This conclusion was further supported by the fact that DNP stimulated remarkably the lysis of an aerobic culture induced by CN^-.

3. Na azide exerted little effect on the aerobic culture at a concentration of $8 \times 10^{-4}\ M$, but caused pronounced lysis at a concentration of 0.01 M. The reason for this difference between the two concentrations was examined. The effect of azide on respiration was therefore determined at both these concentrations (Fig. 1). Respiration was inhibited only at the higher concentration and after some lag period. At the lower concentration, on the

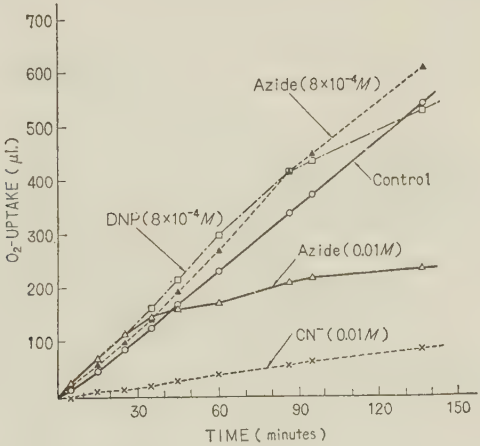

Fig. 1. The effect of various inhibitors on the respiration of *B. subtilis*.

Cells were grown in Soy medium, harvested in the log phase, washed once with salt solution C and resuspended in the following solution: Soluble starch hydrolyzed with crystalline amylase 8 per cent, Na citrate 0.04 M, Na_2HPO_4 0.15 M, KCl 0.02 M, $MgSO_47H_2O$ 0.002 M, $CaCl_2$ 0.001 M, pH 7.2. The composition of the reaction mixture: the cell suspension 1.0 ml., salt solution C 0.5 ml., and the inhibitor solution as described in the figure, total volume 2.0 ml. The inhibitor solution was added to the cell suspension at 0 time and O_2 uptakes were measured.

contrary, it was stimulated. Presumably this is because azide acted in this case as an uncoupler like DNP (*6*). The stimulation by azide of anaerobic lysis at both concentration can be also explained by its uncoupling action as in the case of DNP. In short, the action of azide at the lower concentration ($8\times10^{-4}\,M$) corresponds to that of DNP and the action at the higher concentration ($10^{-2}\,M$) corresponds to the combined action of DNP and CN^-.

4. Chloramphenicol (100 μg./ml.), which was shown to inhibit completely growth and general protein synthesis of this bacterium, had no effect on anaerobic lysis. This result excludes the possibility that anaerobiosis induces the *formation* of the autolytic enzyme. Chloramphenicol did not cause any lysis under aerobic condition either.

The Stability of Cellular Structure and the Phase of Cultural Development—It was reported that in Soy medium which is suitable for amylase production anaerobic lysis is pronounced only in the phase of active amylase production (*1*). The possible relationship between the stability of cellular structure and the exoenzyme production was further examined by measuring the degree of lysis caused by CN^- plus DNP under aerobic conditions at various stages of the culture.

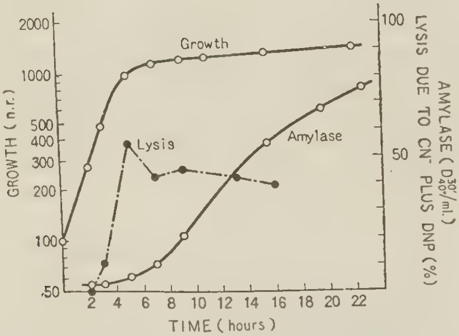

FIG. 2. The lysis caused by CN^- plus DNP at the various phases of cultural development.

Cells were grown in LBY medium for 12 hours at 30° in Monod's shaking apparatus. This culture was diluted (at 0 time) with 20 volumes of fresh Soy medium and shaken further. Both nephelometer readings and amylase determinations were performed periodically. At various times 10 ml. of culture was with drawn, added to 3 ml. of freshly prepared inhibitor solution (NaCN 0.1 M, DNP 0.033 M) and shaken further. Lysis was followed by taking nephelometer readings, and the " per cent lysis " was calculated from the initial readings and the readings after 2 hours.

Cells were grown in Soy medium. At the various phases of the culture development, CN^- plus DNP were added to the culture, which was aerated further. The turbidity of the culture was followed and the degree of lysis was calculated. As shown in figures 2, the lysis due to CN^- plus DNP could not be recognized in the log phase, when amylase was not yet produced, but became pronounced after the cells began to produce amylase. This correlation was also revealed in the experiment when other culture media were used.

Stability of Protoplasts and the Phase of Cultural Development—As already reported (*7*), the addition of polyethylene glycol (PEG) at a high concentration to a medium which is not suited to the good production of amylase, caused the culture to produce much more amylase, and simultaneously, to become more susceptible to anaerobic lysis in the phase of active amylase production. This fact further strengthened the relationship between anaerobic lysis and exoenzyme production. On the other hand, the fact that anaerobic lysis took place even in the presence of a high concentration of PEG

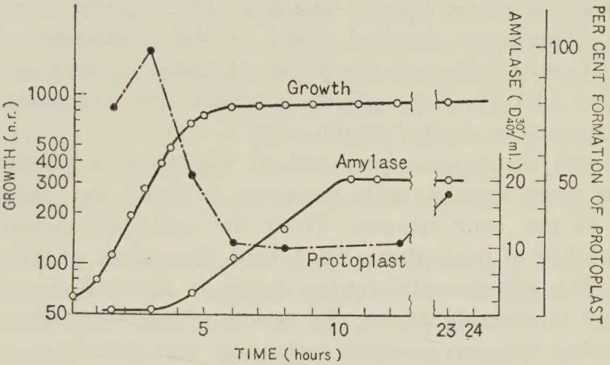

FIG. 3. Amylase formation and stability of protoplasts.

Cells were grown in LBY medium for 18 hours with shaking. This culture was diluted (at 0 time) with 10 volumes of fresh LBY (E, 7.5) medium and shaken further. Both nephelometer readings and amylase determinations were performed periodically. At verious stages, stability of the protoplasts was examined. Cells were collected by centrifugation, washed once with *M*/30 phosphatebuffer (pH 7.2), resuspended in buffer (phosphate *M*/30, pH 7.2) or stabilizing medium (sucrose 15 per cent, phosphate *M*/30, pH 7.2) and treated with lysozyme (10 μg./ml.). From the nephelometer readings after 2 hours, " per cent formations of protoplast " was calculated according to the following equation.

$$\text{" per cent formation of protoplast "} = \left(\frac{N_S - N_{SL}}{N_B - N_{BL}}\right) \times 100$$

N_S: the readings of the suspension in stabilizing medium after 2 hours (expressed as a per cent of initial reading).

N_{SL}: the readings of the suspension treated with lysozyme in stabilizing medium.

N_B: the readings of the suspension treated with lysozyme in buffer medium after 2 hours (expressed in the same way).

N_{BL}: the readings of the suspension treated with lysozyme in buffer medium after 2 hours (expressed in the same way).

suggests that anaerobic lysis involves not only dissolution of cell wall due to the action of the autolytic enzyme, but also destruction of the resulting protoplast. If the anaerobic lysis is the mere dissolution of cell wall by an autolytic enzyme, cells would not lyse but would transform into protoplasts in a protective hypertonic medium (*4*) as in the case of Lederberg's experiment (*8*). Thus, protoplasts appeared to be " unstable " in the phase of active amylase production.

As already described, the cells in the log phase lysed only very slowly. This lysis was prevented by the addition of 12 per cent of PEG. However, the very rapid anaerobic lysis, which occurred in the phase of active amylase production was not protected by PEG. This result supports the above statement that the protoplast is " unstable " in the phase of active amylase production. The more detailed study of the relationship between the " stability " of protoplasts and the phase of cultural development was undertaken using LBY (E, 7.5) medium in which the anaerobic lysis of cells was so pronounced as in Soy medium.

Washed cell suspensions prepared at the various phases of cultural development were treated with lysozyme in both the presence and the absence of 15 per cent sucrose. From the turbidity measurements, the apparent stability of protoplasts, " per cent formation of protoplast ", was calculated. The experimental results shown in figure 3 clearly reveal that the protoplast is unstable during the course of active amylase production. The relationship between amylase formation and instability of protoplasts was also confirmed by direct microscopic examination after the lysozyme treatment.

DISCUSSION

Though many bacteria are known to produce extracellular enzymes, the site of their synthesis is scarcely known. The question, whether exoenzyme is synthesized on the surface of the cell wall or inside the cell wall, has been always posed without any definite answer. Certainly, the demonstration of amylase formation in the cell wall-free protoplast, could provide an unequivocal answer. An approach to this experiment has been made. However, the " protoplasts " prepared from the cells in the phase of active amylase production were unstable as described in the present paper and the demonstration of amylase formation in the " complete " protoplast could not be achieved, though the formation of amylase was demonstrated in the lysed preparation (*9–11*). " Completes " protoplast could be prepared from cells in the log phase grown in LBY (E, 7.5) medium, but even a control intact cell suspension prepared from such a culture could produce only a very small amount of amylase, and therefore production by protoplasts was also very small. However, from repeated experiments of this nature it was concluded that amylase can be formed in the protoplast and increased permeability of the cell membrane described in the present paper also seems to support this statement.

Another experimental approach to this problem is the study of the formation of amylase in a lysate prepared by the action of lysozyme on cells in the phase of active amylase production. The active lysate was prepared in the presence of polyvinyl sulfate and purified protease inhibitor of potato extract and fractionated. Usually the major activity remained with cell residue (precipitate fraction obtained after centrifugation at 900×*g* for 15 minutes). This residue seems to be partially digested cell wall to which are still attached some remaining cellular components (*12*). Unfortunately, in this case, the no clear decision as to the participation of cell wall in amylase formation could be achieved.

In view of various results stated above, amylase may be synthesized at or near the cell membrane and excreted through the cell wall.

Now, as to the mechanism of amylase excretion, the following hypothesis may be advanced taking account of the experimental results obtained so far:

Amylase synthesis is performed at or near cell membrane. An autolytic enzyme is localized very close to the cell wall, and is in an inactive condition. When amylase is excreted, this autolytic enzyme is activated and modifies the sturcture of cell wall, thus rendering the cell wall permeable to amylase molecules.

The mechanism of activation of the autolytic enzyme is not known, but the study of anaerobic lysis suggests some clues. Anaerobic condition or the inhibitors like CN^- and azide ($10^{-2}\,M$) could cause lysis when amylase was being excreted, while an agent like DNP and azide ($10^{-4}\,M$) did not. The actual lysis induced by such procedures may be either the result of the accentuation of the normal activation mechanism, or the result of the inhibition of some regulatory mechanism operating in the course of enzyme excretion. On the other hand, the participation of energy supplying mechanism does not seem to be involved in the activation, though its role for the maintenance of cellular structure was demonstrated.

The autolytic enzyme, which acts on the cell wall (*3*, *4*), is postulated to play a role in enzyme excretion in our strain. It may be generalized to enzyme excretion at large. The mechanism of bacteriophage invasion into host cell proposed by Puck (*13–15*) is analogous to that presented here. Besides, the autolytic enzyme may play a role in normal cellular physiology. It may play a role in the degradation and synthesis of cell wall in the course of normal cellular division (*16*), in the liberation of spores from vegetative cells (*17*) or in the process of transformation into L-form in some bacteria*.

Studies on the detailed mechanism of action of autolytic enzyme involved in such processes seem to be very important to our understanding of general cellular physiology.

SUMMARY

The trigger mechanism by which the autolytic enzyme of *Bacillus subtilis*

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* Personal communication from Dienes.

enters into its action was studied. It was found that cyanide or a high concentration of azide, which inhibit respiration of this bacterium, can cause lysis even under aerobic conditions. 2,4-Dinitrophenol, on the other hand, could not cause lysis, but could stimulate the lysis once the lysis was caused was caused by some trigger mechanism. Chloramphenicol had no effect on anaerobic lysis. Thus, it was concluded that anaerobic lysis may be caused by the activation of a preexisting autolytic enzyme, and this activation may be induced by the reducing condition due to oxygen deficiency.

The relationship between amylase excretion and cellular structure was studied. Lysis due to added cyanide plus 2,4-dinitrophenol was shown to occur only after the culture began to produce amylase. Protoplasts were also shown to be unstable during this phase.

The participation of the autolytic enzyme in the process of enzyme excretion through the cell wall was proposed and discussed.

Authors wish to express their gratitude to Prof. S. Akabori for his encouragement and helpful advice and to thank to Prof. A. Pardee for reviewing the manuscript.

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